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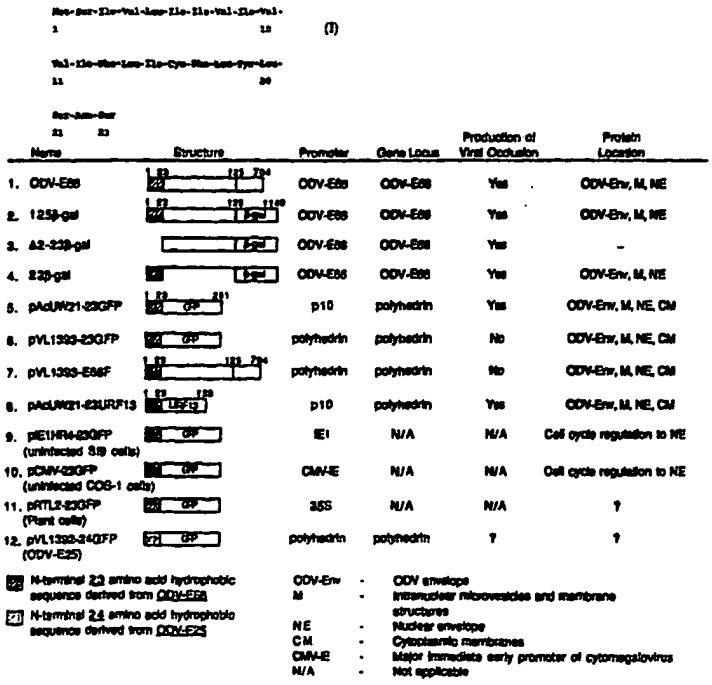
(57) Abstract

The N-terminal region of 23 amino acids of ODV-E66 is composed predominantly of hydrophobic residues and was shown to be necessary and sufficient to target a cytoplasmic marker protein, e.g., beta-galactosidase, green fluorescent protein and URF13, to the intranuclear viral-induced unit-membranes and viral envelopes. The 23 hydrophobic amino acid sequence is (I).



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NUCLEOTIDE AND AMINO ACID SEQUENCE AND USES THEREOF

Background of the Invention

This invention is directed to a nucleotide and amino acid sequence that serves to target foreign proteins to the occlusion derived virus (ODV) envelope, e.g., the N-terminal hydrophobic twenty three (23) amino acid sequence derived from ODV-E66.

I. INTRODUCTION

Naturally occurring insect baculoviruses have received considerable attention for their potential to be used as non-chemical, environmentally safe control agents for insect pests. They infect only arthropods, and each virus strain infects only one or a limited number of species. Many of the virus hosts are commercially important agricultural insect pests. Examples include infestation of cotton, sorghum and corn by the bollworm/budworm complex, and infestation of garden crops by the cabbage looper and tomato hornworm. As important, beneficial insects that are natural predators of insect pests are not hosts for baculoviruses. Baculovirus AcMNPV has undergone 5 controlled release trials and these trials indicate that when the virus is genetically modified by deletion of a single gene, it does not persist in the environment

(Cory and Bishop, 1995). Thus, genetically engineered baculoviruses can be used safely as a "one pass" insecticide that will kill the insect pest and not persist in the environment.

The host range of insect baculoviruses has been extensively studied and no evidence of infection or pathogenic responses have been identified in non-host insects, plants, vertebrates and man (Groner, 1986). This feature may make baculoviruses as ideal agent to be modified and used for the delivery of drugs, genes, or therapeutics. Both the use of baculoviruses as insecticides and as treatment delivery agents in animals and human diseases are discussed, using an amino acid sequence which targets proteins to the viral envelope of the occlusion derived virus (ODV).

ODV is the primary infectious agent to the insect host. The ODV envelope fuses with the plasma membrane of the gut cell, thus releasing the nucleocapsid and viral DNA into the cell (Kawanishi *et al.*, 1972). In this process, viral envelope proteins are incorporated into the gut cell plasma membrane.

A. General Properties of the Baculovirus

The family Baculoviridae consists of viruses with an enveloped rod-shaped virion containing a circular double-stranded DNA genome ranging from 88 to 153 kb (Murphy *et al.*, 1995). Baculoviruses have been isolated only from arthropods; primarily from insects of the order *Lepidoptera*, but also from *Hymenoptera*, *Diptera*, *Coleoptera*, *Neuroptera*, *Thysanura*, and *Trichoptera* as well as from the crustacean order *Decapoda* (shrimp). Individual baculovirus may infect one species or multiple species within the same order (e.g., AcMNPV infection of Lepidopteran). However, none of the baculoviruses infects a large number of insects, or members of different Orders of the Class Insecta. *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) is unusual among baculoviruses because it displays a wider host range than most baculoviruses, which includes 32 species in 12 families (Martignoni *et al.*, 1982).

AcMNPV is the most extensively studied baculovirus and its genome sequence is known (Ayres *et al.*, 1994). It is distinguished by a unique biphasic life cycle in its lepidopteran host insect (reviewed in Blissard and Rohrmann, 1990). Infection produces high titers of two forms of progeny virus, budded virus (BV) and ODV. Both forms are essential for natural propagation of the virus. Infection initiates when the insect feeds on a food source contaminated by viral occlusions. The alkaline midgut fluids dissolve the viral occlusion, and release ODV to infect the midgut epithelium. Early in infection nucleocapsids assemble in the nucleus, migrate to the cell surface, and obtain an envelope by budding through the plasma membrane into the insect hemolymph. This form of virus is named BV and is responsible for secondary or systemic infection of a variety of tissues in the insect. Late in infection the production of BV decreases. Instead of budding from the cell surface, the nucleocapsids remain within the nucleoplasm where they are enveloped and occluded within the viral occlusion. This form of virus is named occlusion derived virus or "ODV." The insect midgut epithelium is generally believed to be the site of primary infection *in vivo* (Adams and McClintock, 1991).

ODV and BV contain the same genome, yet exhibit significant differences in morphology, timing and cellular site of maturation, structural proteins, source of viral envelopes, antigenicity, and infectivity (Blissard and Rohrmann, 1990; Braunagel and Summers, 1994). The differences between the envelopes of ODV and BV may function to regulate important aspects of the role of these two viral forms in infectivity, host range, and tissue specificity.

B. Regulation of Viral Gene Expression

AcMNPV gene expression is temporally regulated. AcMNPV genes are classified into two phases (early and late) which are further subdivided into four classes: immediate early or "IE", delayed early, late, and very late (Blissard and Rohrmann, 1990). Promoters of immediate early genes are active in uninfected insect

cells and are expressed very early in infection, thus making them ideal promoters for the expression of proteins with insecticidal properties to compromise infected insects at a very early stage or for the expression of genes in transiently transfected or stably transformed cells. Five immediate early (IE) genes have been identified, e.g., IE-0 (Chisholm and Henner, 1988) and IE-1 (Kovacs *et al.*, 1991a, b; Choi and Guarino, 1995a, b, c), IE-2 (Carson *et al.*, 1988, 1991), PE-38 (Krappa *et al.*, 1992), and ME53 (Morsdorf *et al.*, 1993). Very late genes are highly expressed very late during infection and their promoters have been utilized to express a variety of proteins using the baculovirus expression vector system. Two very late genes are known: polyhedrin (Hooft van Iddekinge *et al.*, 1983) and p10 (Kuzio *et al.*, 1984). The identity and nucleotide sequence of promoters representing all classes of genes are known and can be used for the expression of chimeric genes attached to them or simultaneous expression of multiple genes at controlled times.

C. **Virion Structure**

1. Nucleocapsid

The differences in nucleocapsid protein composition between ODV and BV of AcMNPV have been investigated (Braunagel and Summers, 1994). Several proteins have been identified as common nucleocapsid components in both phenotypes of AcMNPV (Blissard and Rohrmann, 1990; Rohrmann, 1992), including: p39 (Thiem and Miller, 1989); p80 (Lu and Carstens, 1992); p6.9 (Wilson *et al.*, 1987; Wilson, 1988); p24 (Wolgamot *et al.*, 1993). Homologs of these nucleocapsid proteins have also been identified in other baculoviruses.

2. Viral envelope

Generally, the ODV envelope is closely associated with the nucleocapsid and has no apparent specialized morphology, whereas the BV envelope is loose-fitting and has a specialized region (peplomer) at one end of the virion which is enriched with the viral envelope glycoprotein gp64 (Adams and McClintock, 1991).

The biochemical composition of both viral envelopes is fairly well defined. A recent study shows major differences between BV and ODV in envelope protein SDS-PAGE profiles, major antigens, lipid, and fatty acid compositions (Braunagel and Summers, 1994).

a. BV envelope proteins

So far, only one BV envelope protein (gp64) has been mapped in the genomes of AcMNPV (Whitford *et al.*, 1989), OpMNPV: *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus (Blissard and Rohrmann, 1989), and CfMNPV: *Christoneura fumiferana* multinucleocapsid nuclear polyhedrosis virus (Hill and Faulkner, 1994). Gp64 is a type I transmembrane protein which is N-glycosylated (Rohrmann, 1992).

AcMNPV encodes a ubiquitin-like protein which is integrated into the BV envelope through a novel type of phospholipid anchor (Guarino *et al.*, 1995).

The baculovirus apoptotic inhibitor p35 is also detected in purified BV by Western-blot analysis; however, the exact location of p35 in the virion is unknown. It is not known whether p35 is present in ODV (Hershberger *et al.*, 1994).

b. ODV envelope proteins

Several ODV envelope proteins of AcMNPV have been identified, including ODV-E66 (Hong *et al.*, 1994), ODV-E56 (Braunagel *et al.*, 1996a), and ODV-E18/E35 (Braunagel *et al.*, 1996b). Other candidate ODV envelope proteins have also been identified. A fatty acylated protein of 26.4 kDa (p26.4) is associated with AcMNPV ODV (Rohrmann, 1992). The p74 protein is known to play a role in ODV infectivity (Kuzio *et al.*, 1989) and is believed to be a ODV structural protein; however, its location within the virus has not been determined. Two ODV envelope proteins have been identified in other baculoviruses: Vp17 of *Plodia interpunctella* granulosis virus (Funk and Consigli, 1993) and ODV-E25 (or p25) of *Orygia*

pseudotsugata multinucleocapsid nuclear polyhedrosis virus (Russell and Rohrmann, 1993).

3. Tegument

An ODV-specific structural O-linked glycoprotein, gp41, is suggested to reside between the envelope and nucleocapsid, ie. the tegument (Whitford and Faulkner, 1992a, b).

D. Replication pathway of AcMNPV

The replication pathway of AcMNPV is briefly discussed below. A more extensive review can be found in Adams and McClintock (1991).

1. Virion attachment and penetration

Two routes, adsorptive endocytosis (or viropexis) and direct fusion of BV envelope with plasma membrane, are initially proposed for entry of BV into cultured cells. Although BV may enter cells by fusion (Volkman *et al.*, 1986; Kozuma and Hukuhara, 1994), the majority of data indicates that the primary route is by adsorptive endocytosis (Charlton and Volkman, 1993).

By extrapolation with other enveloped animal viruses, especially orthomyxoviruses (White, 1990), a model is proposed to explain the BV entry process in cultured insect cells (reviewed in Volkman, 1986). According to this model, BV virions enter cells via the endocytic pathway during which virions are invaginated into the cytoplasm as clathrin-coated vesicles. In the cytoplasm, the clathrin coat dissociates and the uncoated vesicles containing virions fuse with cellular vesicles to form endosomes. Following the decrease of pH in the endosome, presumably by an ATP-driven proton pump, the BV envelope fuses with the endosomal membrane through the function of gp64, and release the nucleocapsid into the cytoplasm.

The entry of ODV into the midgut epithelial cells is believed to occur through the fusion of the ODV envelope with the

plasma membrane of the midgut cells (reviewed in Adams and McClintock, 1991).

2. Nucleocapsid uncoating

After nucleocapsids are released into the cytoplasm, they are transported to the nucleus for uncoating.

3. Progeny nucleocapsid assembly

Progeny nucleocapsids are assembled within the nucleoplasm of infected cells.

4. Envelopment of progeny viruses

The envelopes of ODV and BV are derived from distinct cellular sources and appear to exhibit marked tissue specificity (Adams and McClintock, 1991). ODV appears to be specialized both for interaction with polyhedrin and for infection of the epithelial cells in the insect midgut. BV is specialized for interaction with other cell types and tissues in the insect. ODV is responsible for spreading infection among the natural insect hosts, whereas BV is responsible for the secondary infection within the infected host and in cultured insect cells. There are dramatic differences in the infectivity of ODV and BV both *in vivo* and *in vitro* as well as in their ability to be neutralized by homologous and heterologous antisera (Volkman *et al.*, 1976; Volkman and Summers, 1977; Keddie and Volkman, 1985). Differences in infectivity correlate with the different roles of these two viral forms in the virus life cycle (Blissard and Rohrmann, 1990).

In the early phase, nucleocapsids destined for BV escape the nucleus, possibly by budding through both outer and inner nuclear membranes, and reach the plasma membrane in an unenveloped state. Then, nucleocapsids bud through the plasma membrane to become BV virions. Budding appears to occur at gp64-containing plasma membrane regions with a "spiny coat" appearance. BV also contains a "spiny coat" structure called peplomer at one end of the virion. In the mature BV, gp64 is distributed throughout the envelope, but

appears more concentrated in the peplomer, thus constituting the major protein component of the peplomer.

In the late phase, ODV are enveloped from membrane structures, microvesicles, induced within the nucleoplasm of infected cells. Intranuclear morphogenesis of microvesicles begins at approximately 12 h. p.i. The microvesicles have a unit membrane structure and they normally appear as open-ended membranes as well as thin tubular membranes. ODV nucleocapsids display polarity in their association with microvesicles. In many cases, before the completion of envelopment, they are seen lined with each other with the capped end attached to the membrane. After ODV obtains its envelope, it is further occluded into the viral occlusion which is released after cell lysis and is very stable in the environment for many years.

Maturation of the ODV envelope within the nucleus is very unique compared to herpes simplex virus (HSV) and plant nucleohabdovirus (Jackson *et al.*, 1987; Roizman and Sears, 1990). Maturation of HSV results in initial envelopment of nucleocapsids in the perinuclear space, but the progeny viruses are further transported through the cytoplasm to the exterior of the cell (Roizman and Sears, 1990). In contrast, there is no evidence that ODV ever leaves the nucleus for ODV envelope proteins to be further modified in the cytoplasm after their incorporation into the ODV envelope. During HSV infection, the inner nuclear membrane is modified in regions where nucleocapsids bud, but unit-membranes are not observed in the nucleoplasm. In contrast, baculoviruses induce extensive elaboration of unit-membranes within the nucleoplasm and budding of nucleocapsids through unit-membranes occurs at the intranuclear membranes.

Summary of the Invention

In general, a novel polypeptide, designated 23AA, is identified and characterized. The 23AA polypeptide is the N-terminal 23 amino acids of ODV-E66 and includes a hydrophobic domain. This nucleotide sequence encodes for a transport polypeptide which directs the localization of fused desired protein(s) to occlusion derived virus envelopes.

For this invention, the phrase "transport polypeptide" can also be described as a "retention polypeptide," as a "target polypeptide," or as a "signal polypeptide." In this respect, "transport polypeptide" is meant to include a "retention polypeptide," a "target polypeptide" and a "signal polypeptide."

This amino acid sequence can serve as a targeting signal, as shown with β-galactosidase (β-gal), green fluorescent protein (GFP) and URF-13 protein, for incorporating many different proteins, having potentially any function, into the occlusion derived viral (ODV) envelope.

This small amino acid sequence has been fused to a number of proteins, all with differing sites of localization, e.g., β-gal (a protein that is normally cytoplasmic), green fluorescent protein (GFP; a protein that usually resides in both the cytoplasm and nucleus) and URF-13 (a protein that normally resides within the plasma membrane). The experimental data confirms that these marker proteins are targeted and reside within the ODV envelope of the recombinant viruses and intranuclear viral-induced microvesicles. Prior to this invention, there were no known methods to accomplish protein targeting to the ODV envelope and intranuclear microvesicles.

The N-terminal 23 amino acids are composed of the following amino acids:

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Met-Ser-Ile-Val-Leu-Ile-Ile-Val-Ile-Val-Val-Ile-Phe-Leu-Ile-
1               5                  10                  15

Cys-Phe-Leu-Tyr-Leu-Ser-Asn-Ser
            20          23
```

The invention includes a substantially pure DNA encoding a transport polypeptide, a strand of which DNA will hybridize at high stringency to a probe containing a sequence of at least 15 consecutive nucleotides of Fig. 1B (23AA). The protein encoded by the DNA of the invention may share at least 80% sequence identity (preferably 85%, more preferably 90%, and most preferably 95%) with the amino acids listed in Fig. 1B

More preferably, the DNA includes the coding sequence of the nucleotides of Fig. 1B, or a degenerate variant of such a sequence. Also included in this invention, is isolated DNA having a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of Fig. 1B; and (b) nucleotide sequences which, through the degeneracy of the genetic code, encode the same peptide gene product as that encoded by the nucleotide sequence of Fig. 1B.

The probe to which the DNA of the invention hybridizes preferably consists of a sequence of at least 20 consecutive nucleotides, more preferably 40 nucleotides, and most preferably 50 nucleotides, of the coding sequence of the nucleotides listed in Fig. 1B or the complement thereof. Such a probe is useful for detecting expression of 23AA transport polypeptide in a biological sample by a method including the steps of (a) contacting a biological sample with an antibody or antibody fragment made against 23AA, and (b) determining whether the antibody or antibody fragment binds to a component of the sample, such binding being an indication that the sample contains 23AA transport polypeptide having a hydrophobic domain.

This invention also includes a substantially pure DNA containing a sequence of at least 15 consecutive nucleotides (preferably 20, more preferably 30, even more preferably 50, and most preferably all) of the region from nucleotides 122 to 190 of the nucleotides listed in Fig. 1A.

By "high stringency" is meant DNA hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65°C at a salt concentration

of approximately 0.1 x SSC, or the functional equivalent thereof. For example, high stringency conditions may include hybridization at about 42°C in the presence of about 50% formamide; a first wash at about 65°C with about 2 x SSC containing 1% SDS; followed by a second wash at about 65°C with about 0.1 x SSC.

By "substantially pure DNA" is meant DNA that is not part of a milieu in which the DNA naturally occurs, by virtue of separation (partial or total purification) of some or all of the molecules of that milieu, or by virtue of alteration of sequences that flank the claimed DNA. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by polymerase chain reaction (PCR) or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence, e.g., a fusion protein. Also included is a recombinant DNA which includes a portion of the nucleotides listed in Fig. 1A which encodes an alternative splice variant of 23AA.

The DNA may have at least about 70% sequence identity to the coding sequence of the nucleotides listed in Fig. 1B, preferably at least 75% (e.g. at least 80%); and most preferably at least 90%. The identity between two sequences is a direct function of the number of matching or identical positions. When a subunit position in both of the two sequences is occupied by the same monomeric subunit, e.g., if a given position is occupied by an adenine in each of two DNA molecules, then they are identical at that position. For example, if 7 positions in a sequence 10 nucleotides in length are identical to the corresponding positions in a second 10-nucleotide sequence, then the two sequences have 70% sequence identity. The length of comparison sequences will generally be at least 30 nucleotides, and most preferably at least 50 nucleotides. Sequence identity is typically measured using sequence analysis software (e.g., Sequence

Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

The invention also includes a vector containing a DNA encoding a polypeptide which includes the amino acid sequence of Fig. 1B, e.g., a construct in which the coding sequence is operably linked to a suitable promoter or other regulatory sequences for expression of the polypeptide, and a cell containing such a vector. This vector includes a suitable promoter that is compatible to the system used and directs the expression of the desired protein. The cell may be procaryotic or eukaryotic and preferably expresses the recombinant polypeptide encoded by the nucleotides listed in Fig. 1B.

A "vector" is defined as a replicable nucleic acid construct, e.g., a plasmid or viral nucleic acid. Vectors may be used to amplify and/or express nucleic acid encoding 23AA protein. An expression vector is a replicable construct in which a nucleic acid sequence encoding a polypeptide is operably linked to suitable control sequences (promoters) capable of effecting expression of the polypeptide, e.g., 23AA, in a cell. The need for such control sequences will vary depending upon the cell selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter and/or enhancer, suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Methods which are well known to those skilled in the art can be used to construct expression vectors containing appropriate transcriptional/translational control signals. See for example, the techniques described in Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual (2nd Edition), Cold Spring Harbor Press, N.Y., which are incorporated by reference. A gene and its transcription control sequences are defined as being "operably linked" if the transcription control sequences effectively control the transcription of the gene. Vectors of the invention include, but are not limited to, baculovirus expression vectors and other viral vectors. Preferred viral vectors of the invention are those derived

from retroviruses, adenovirus, adeno-associated virus, SV40 virus, or herpes viruses.

As stated above, the invention features a cell preferably expressing the recombinant polypeptide encoded by the nucleotides listed in Fig. 1B. This cell can be a prokaryotic cell, e.g., an *Escherichia coli* cell, or a eukaryotic cell. Eukaryotic cells that can be used in the invention include, but are not limited to, COS, CHO, HeLa, TN368, Sf21, and Sf9 cells. In the case of a eukaryotic cell, the gene may or may not be integrated into the genome of the cell. Also included in the invention is an essentially homogeneous population of prokaryotic or eukaryotic cells, each of which contains (i.e., is transfected with) a recombinant 23AA gene. Transfection can be transient or stable, and if desired can be carried out *in vivo* or *ex vivo*, using the patient's own cells.

The invention also includes a substantially pure DNA 23AA protein (a) containing a hydrophobic domain; and (b) localizing to occlusion derived virus envelopes thereby directing the localization of fused or hybrid proteins to occlusion derived virus envelopes.

Preferably, the 23AA transport polypeptide includes the amino acid sequence of Fig. 1B, e.g., in the form of a 23AA fusion protein. By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). The amino acid sequence of the transport polypeptide preferably differs from the amino acid sequence of Fig. 1B solely by conservative amino acid substitutions, e.g., substitution of one amino acid for another of the same class (e.g., valine for alanine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence where the changes do not destroy the function of the transport polypeptide (e.g., to direct the localization of fused or hybrid proteins to occlusion derived virus envelopes).

Preferably, the amino acid sequence of the hydrophobic transport polypeptide, 23AA, is at least 80%, more preferably 85%,

more preferably 90%, and most preferably 95% identical to the amino acid sequence of Fig. 1B.

By a "substantially pure transport polypeptide" is meant a polypeptide which has been separated from at least some of those components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and other naturally-occurring organic molecules with which it is naturally associated in vivo. Preferably, the purity of the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight. A substantially pure 23AA polypeptide may be obtained, for example, by extraction from a natural source (e.g., baculovirus ODV-E66); by expression of a recombinant nucleic acid encoding an 23AA polypeptide; or by chemically synthesizing the polypeptide. Purity can be measured by any appropriate method, e.g., column chromatography such as immunoaffinity chromatography using an antibody specific for 23AA, polyacrylamide gel electrophoresis, or HPLC analysis. A protein or polypeptide is substantially free of naturally associated components when it is separated from at least some of those contaminants which accompany it in its natural state. Thus, a polypeptide which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be, by definition, substantially free from its naturally associated components. Accordingly, substantially pure proteins or polypeptides include eukaryotic proteins synthesized in *E. coli*, other prokaryotes, or any other organism in which they do not naturally occur.

Also included in this invention is substantially pure DNA that includes a sequence of at least 15 consecutive nucleotides of substantially pure DNA from the region of nucleotides 122 to 190 of Fig. 1A.

Also, this invention includes substantially pure DNA that includes a sequence of at least 15 consecutive nucleotides of substantially pure DNA encoding the transport polypeptide 23AA and

defined as nucleotides 122 to 190 of Fig. 1A. The amino acid sequence of this transport polypeptide is:

M S I V L I I V I V V I F L I C F L Y L S N S.

The invention also includes fragments (e.g., antigenic fragments) of the 23AA polypeptide. As used herein, "fragment," as applied to a polypeptide, will ordinarily be at least 10 residues, more typically at least 20 residues in length. Fragments of the 23AA polypeptide can be generated by methods known to those skilled in the art, e.g., by enzymatic digestion of naturally occurring or recombinant 23AA polypeptide, by recombinant DNA techniques using an expression vector that encodes a defined fragment of 23AA, or by chemical synthesis. The ability of a candidate fragment to exhibit a characteristic of 23AA (e.g., binding to an antibody specific for 23AA) can be assessed by methods described herein. Purified 23AA or antigenic fragments of 23AA can be used to generate new antibodies or to test existing antibodies (e.g., as positive controls in a diagnostic assay) by employing standard protocols known to those skilled in the art. In one preferred embodiment, a monoclonal antibody is generated using the hydrophobic domain which corresponds to an amino acid sequence of Fig. 1B to immunize an appropriate laboratory animal, such as a mouse. Also included in this invention are polyclonal antisera generated by using 23AA or a fragment of 23AA as the immunogen in, e.g., rabbits. Standard protocols for monoclonal and polyclonal antibody production known to those skilled in this art may be employed. The monoclonal antibodies generated by this procedure can be screened for the ability to identify recombinant 23AA cDNA clones, and to distinguish them from known cDNA clones.

Also included in the invention are 23AA polypeptides which are encoded at least in part by portions of Fig. 1B, e.g., products of alternative mRNA splicing or alternative protein processing events, or in which a portion of 23AA sequence has been deleted or altered. The fragment, or the intact 23AA polypeptide, may be

covalently linked to another polypeptide, e.g. which acts as a label, a ligand or a means to increase antigenicity.

The invention also includes a polyclonal or monoclonal antibody which specifically binds to 23AA. Preferably, this antibody specifically binds to an epitope in the hydrophobic domain of 23AA which corresponds to the sequence shown in Fig. 1B. The invention encompasses not only an intact monoclonal antibody, but also an immunologically-active antibody fragment, e.g., a Fab or $(Fab)_2$ fragment; an engineered single chain Fv molecule; or a chimeric molecule, e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and the remaining portions of another antibody, e.g., of human origin.

In preferred embodiments, the antibody, or a fragment thereof, may be linked to a toxin or to a detectable label, e.g. a radioactive label, non-radioactive isotopic label, fluorescent label, chemiluminescent label, paramagnetic label, enzyme label, or colorimetric label.

Examples of suitable toxins include diphtheria toxin, *Pseudomonas* exotoxin A, ricin, and cholera toxin.

Examples of suitable enzyme labels include malate hydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholinesterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, etc.

Paramagnetic isotopes for purposes of *in vivo* diagnosis can also be used according to the methods of this invention. There are numerous examples of elements that are useful in magnetic resonance imaging. For discussions on *in vivo* nuclear magnetic resonance imaging, see, for example, Schaefer et al., (1989) *JACC* 14, 472-480; Shreve et al., (1986) *Magn. Reson. Med.* 3, 336-340; Wolf, G. L.,

(1984) *Physiol. Chem. Phys. Med. NMR* 16, 93-95; Wesbey et al., (1984) *Physiol. Chem. Phys. Med. NMR* 16, 145-155; Runge et al., (1984) *Invest. Radiol.* 19, 408-415.

Examples of suitable fluorescent labels include a fluorescein label, an isothiocyalate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an ophthaldehyde label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, an aequorin label, etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy et al., (1976) *Clin. Chim. Acta* 70, 1-31; and Schurs et al., (1977) *Clin. Chim. Acta* 81, 1-40. Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method. All of these methods are incorporated by reference herein.

Also within this invention, is a method of directing a desired protein to occlusion derived virus envelopes which includes the steps of: (a) constructing a vector comprising a marker protein, e.g., beta-galactosidase, and the DNA of claim 2; (b) fusing a nucleotide sequence encoding a desired protein to the DNA of Step (a) to form a hybrid vector; (c) incubating the hybrid vector of Step (b) with a biological sample; and (d) determining the localization of the marker protein within the biological sample, wherein localization indicates the presence of occlusion derived virus envelopes and also delivery of the hybrid vector to the envelopes.

USES AND ADVANTAGES

As described herein, this invention, an N-terminal twenty three amino acid (23AA) hydrophobic sequence, capable of targeting desired proteins to the ODV envelope, provides a number of uses and advantages. In addition to the many uses and advantages described above, this unique 23AA sequence can be used in the following ways.

For example, the 23AA sequence can be used to target desired proteins, with known properties or desired characteristics, to the ODV envelope.

This invention will also be useful in identifying the signals and pathways for the transport and assembly of membrane proteins into the nucleus and/or the use of specific targeting signals from ODV-E66 to locate potentially any protein, peptide, or derivatives to the nuclear envelope or other nuclear membranes.

Additionally, this 23AA sequence of this invention can be used to locate a protein, peptide or derivative of such (23AA-protein product) to cytoplasmic membranes, the nuclear envelope, the inner nuclear membrane or intranuclear membranes induced during viral infection or during specific stage(s) of cell cycle.

Examples of insect infected cells, include, but are not limited to (a) Sf9 cells, Sf21 cells or related strains, (b) TN368 cells or related strains or (c) any insect cell (or invertebrate cell) infected by a baculovirus (e.g., AcMNPV) expressing a structural or functional protein cognate containing the 23AA sequence or its structural/functional equivalent.

Examples of viral infection of insects include, but are not limited to (a) *Heliothis virescens* larvae or related susceptible strains; (b) *Trichoplusia ni* larvae or related susceptible strains; or (c) any insect larvae species susceptible to infection by a baculovirus.

The use of the claimed 23AA sequence for directing the location of 23AA-protein product in a desired cell would include, but not limited to, the delivery of proteins, peptides or derivatives thereof for (a) insecticidal effects - insect pest control; (b) therapeutic applications (e.g., the localization of a protein,

peptide or derivative into the nuclear envelope which will alter the cell cycle); and (c) diagnostics (e.g., insert a marker specific for a disease or abnormality into a cell tissue manifesting such abnormalities).

Expression of the 23AA-protein Product in Infected Cells

The 23AA-protein product of this invention can be expressed in infected cells as a single copy, multiple copies of the same protein or in variable combinations with other proteins (e.g., 23AA-protein A, 23AA-protein B, ... 23AA-protein X). The 23AA-protein product of this invention can also be expressed in the infected cell at any time during the infection process by expression from promoter classes representing immediate early (0-4 hpi), delayed early (4-6 hpi), late (6-24 hpi) or very late (24 hpi-5 days or more).

Also, the 23AA-protein product can be directed to locate to the desired locations in vertebrate, plant or other cell types during viral infection of that organism or cell. This can be done by placing the 23AA-protein product under the expression of any viral gene of any viral promoter class engineered for expression during infection of vertebrate, plant or microbial cells. This could therefor introduce the 23AA-protein product into the cell during the infection cycle to deliver the desired protein into cytoplasmic or nuclear membranes.

Expression of the 23AA-protein Product in Uninfected Cells

Additionally, any 23AA-protein product can be expressed in uninfected cells by using gene promoters that function in cells in the absence of or during viral infection. For example, the desired 23AA-protein product construct can be expressed in uninfected insect cells by the AcMNPV-IE1 or other immediate early gene promoters regulated by the cell DNA-dependent RNA polymerase; or, expressed from a baculovirus (or other) gene promoter transactivated by an

immediate early gene; or, a host cell gene promoter which is constitutively expressed, cell cycle or developmentally regulated.

Examples of transfected, but uninfected cells, include, but are not limited to, (a) Sf9 cells or related strains (Sf21, etc.); (b) TN368 cells or related strains; and (c) any insect or invertebrate cells, vertebrate cells, plant cells, or microbial cells capable of transfection.

Also, examples of transformed, but uninfected cells, include, but are not limited to, (a) Sf9 cells or related strains (Sf21, etc.); (b) TN368 cells or related strains; and (c) any insect cells, invertebrate cells, vertebrate cells, plant, or microbial cells capable of transformation.

Additionally, the 23AA-protein product taught in this invention can be delivered to insect cells, insects, vertebrates, plant and microbial cells or organisms for targeting to the endoplasmic reticulum, nuclear envelope and intranuclear membranes by a variety of artificially developed or genetically engineered strategies. For example:

(1) The use of artificial membranes (liposomes) to deliver 23AA-protein product to the cell surface which could be then targeted to ER and nuclear envelope by the cellular endocytic and vesicular retrograde transport pathways

(2) A retrovirus engineered to target a specific cell or tissue type (e.g., retroviruses), could carry in the viral envelope a specific 23AA-protein product or, have incorporated in the viral genome sequence, information specific for expression, translation and targeting of the derived 23AA-protein product during or after germline transformation of the cell.

(3) The nucleotide sequence for a derived 23AA-protein product could be incorporated into a protein or polyprotein which would be processed at a specific time in the cellular or viral protein processing pathway for incorporation into cytoplasmic membranes, nuclear envelope, or induced nuclear membranes.

Also, there are many possibilities for deriving or obtaining a 23AA sequence or its structural and functional cognate for any of the uses as described above. For example, the 23AA sequence may be derived from, for example, but not limited to: (1) the ODV-E66 gene sequence; (2) a synthetic DNA sequence with codon usage for a particular cell species or organism optimized for the 23AA. This would include the use of codons not preferred by the baculovirus nucleotide sequence which would translate the same amino acid because of degeneracy in the code; (3) a synthetic 23AA peptide or a structural and functional cognate of amino acids made synthetically or derived in part or whole from another naturally occurring protein sequence; (4) the ODV-E66 genes or proteins of other baculoviruses; and (5) the ODV-E25 protein from any baculovirus with a similar hydrophobic sequence.

DEFINITIONS

For the purpose of this invention, certain phrases and words are defined or used as follows:

Purified polyhedra and viral occlusions are used interchangeably. Polyhedra purified from insect larva are stable for years.

Polyhedra derived virus or "PDV" and occlusion derived virus or "ODV" are used interchangeably.

By "BEVS" is meant baculovirus expression vector system.

By "h p.i." or "hr p.i." is meant hours post infection.

By "β-gal" is meant beta-galactosidase and these terms are used interchangeably.

* * *

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1A is a diagram showing the nucleotide and amino acid sequences of ODV-E66.

Fig. 1B is a diagram showing the nucleotide and amino acid sequences of the N-terminal 23 amino acid of ODV-E66 (nucleotide numbers 122 to 190).

Fig. 2 is an outline summarizing the different constructs, e.g., Construct #1 - Construct #12, used in this invention.

Fig. 3A, 3B and 3C are photographs showing immunogold labeling of purified ODV and wild type AcMNPV infected Sf9 cells at 48 hr p.i.

Figs. 4A, 4B, and 4C are photographs showing immunogold labeling of Sf9 cells infected with pVL1393-E66F at 48 hr p.i.

Figs. 5A and 5B are photographs showing immunogold labeling of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (TN368) cells infected with 23β-gal virus at 48 hr p.i.

Figs. 6A-6I are photographs showing localization of 23GFP in pVL1393-23GFP infected Sf9 cells.

Figs. 7A and 7B are photographs showing immunogold labeling of Sf9 cells infected with pAcUW21-23URF13 virus at 48 hr p.i.

Figs. 8A-8H are photographs showing transient transfection of uninfected Sf9 cells with pIE1HR4-23GFP.

Figs. 9A and 9B are photographs showing fluorescence microscopy of mammalian COS-1 cells transiently transfected with pCMV-23GFP.

Figs. 10A and 10B are diagrams showing an overview of directed delivery to target cell and subsequent gene delivery.

Fig. 11 is a diagram showing levels of protein production utilizing BEVS technology and comparing amounts of foreign protein produced in tissue culture and larva.

Fig. 12 is genomic map of *Autographa californica* NPV showing a construct containing dual promoters and which is occlusion positive and contain both NT-URF13 and WT t-URF13.

Fig. 13 is a chart showing potential applications for the 23AA sequence as described in the Examples.

Brief Description of the Tables

Table 1 outlines how the 23AA hydrophobic sequence of this invention functions differently from the other reported non-baculovirus inner nuclear membrane proteins.

Table 2 outlines known hydrophobic sequences identified in baculovirus ODV envelope proteins.

Detailed Description

AcMNPV nuclear maturation serves as a unique system to study membrane biogenesis in the nucleus and current research indicates the involvement of the membranes of the nuclear envelope in this process. Study of this process may yield insights into the biogenesis of the membranes of the nuclear envelope and, in a broader sense, help to understand how eukaryotic cells assemble their elaborate network of intracellular membranes. Understanding the molecular basis of the synthesis, trafficking and assembly of ODV envelope proteins into the nucleus is of utmost importance. Marker proteins specific to the ODV envelope have been identified, one of which is ODV-E66. Thus, these experiments were designed:

1) To identify the gene encoding ODV-E66 and analyze its transcriptional and translational expression;
2) To study its function in ODV nuclear maturation;
3) To investigate the nuclear source of intranuclear viral-induced membranes
4) To study ODV envelope protein synthesis, trafficking and assembly into intranuclear viral-induced membranes with the aim to identify specific protein targeting signals and viral or cellular transport factors;
5) To reduce to practice the identified targeting signals in a variety of insecticidal, animal, and human health applications.

ODV-E66 is an Integral Membrane Protein of the ODV Envelope

A baculovirus late gene encoding a 66 kDa structural protein (ODV-E66) of AcMNPV has been mapped, cloned, sequenced, and its expression in infected cells studied. ODV-E66 is an integral membrane protein of the ODV envelope. In the infected cell nucleus, ODV-E66 is present in the intranuclear viral-induced microvesicles and ODV envelope, providing evidence that the microvesicles function as an immediate precursor in the assembly of the ODV envelope.

In infected cells, ODV-E66 localizes to the ODV envelope, intranuclear microvesicles, membranes of the nuclear envelope, and cytoplasmic membranes in close juxtaposition to the nuclear envelope. A mutant virus in which the ODV-E66 gene was replaced with a fusion gene encoding the N-terminal 125 amino acids of ODV-E66 and *E. coli* β-galactosidase (β-gal) showed that the 125-amino acid N-terminal domain targeted the β-gal fusion protein to the intranuclear microvesicles and ODV envelope. This invention specifically deals with a unique N-terminal hydrophobic sequence of 23 amino acids which is derived from the N-terminal 125 amino acids of ODV-E66. In infected insect cells this 23-amino acid sequence targets β-gal, green fluorescent protein (GFP), and URF-13 to the membranes of the nuclear envelope which are further transported into the intranuclear microvesicles and ODV envelope.

In uninfected insect and mammalian (COS-1) cells, this sequence targets GFP to the membranes of the nuclear envelope. This amino acid sequence also inserted β-gal into microsomal membranes in the rabbit reticulocyte *in vitro* translation system. This is the first report of a unique sequence which can specifically target foreign proteins to the nuclear envelope, intranuclear microvesicles, and ODV envelope and has important applications in insecticide, human, and animal health.

Unique Features of the 23AA Sequence of this Invention Not Found in Other Membrane Proteins

Listed below is a summary of known hydrophobic protein sequences of inner nuclear membrane proteins reported in the literature (see Table 1 below). Except for HSV-1 gB (protein #2 below), all of the proteins listed in Table 1 below are non-viral proteins. Also, all of these proteins are located in either the nuclear envelope, the nuclear pore, and/or cytoplasmic membrane. Of interest, except for the rat gp210 protein, all of the other listed proteins localize to the inner nuclear membrane (INM).

The Table 1 below outlines how the 23AA hydrophobic sequence of this invention functions differently from the other reported non-

baculovirus inner nuclear membrane proteins. Specifically, in infected cells, the 23AA sequence of this invention targets proteins to intranuclear viral-induced membranes, in addition to protein targeting to nuclear envelope and cytoplasmic membranes.

Also, in uninfected Sf9 and COS-1 cells, the targeting of proteins to membranes of the nuclear envelope (NE) correlates with cell cycle-regulated movement of integral membrane proteins.

The following abbreviations are used in Table 1: **LBR** - Lamin B receptor; **INM** - Inner nuclear membrane; **TM** - Transmembrane domain; **NE** - Nuclear envelope; **HSV** - Herpes simplex virus; **gB** - Glycoprotein B; **ER** - Endoplasmic reticulum; **LAP** - Lamina associated protein; and **NPC** - Nuclear pore complex.

Table 1: Inner Nuclear Membrane Proteins

Protein	Location	Summary
Chicken LBR (COS-7 cells) Smith and Blobel, 1993 and Soullam and Worman, 1993	INM (not detected on ER, Golgi, and cell surface)	1. Chicken LBR has 8 potential transmembrane segments. The first segment is from #206-226. TM: FGTFMLMFFLPATVLYLVLMC 2. Region from #205-246, including the first TM, when fused to the C-terminus of β-gal, is sufficient to target fusion protein to INM. 3. The nucleoplasmic domain (191 aa) of LBR, when fused to the TM and C-terminal domain of chicken heptic lectin (a type II protein with internal signal sequence-the TM), targets the fusion protein to INM. The nucleoplasmic domain of LBR, when expressed alone, is in the nucleoplasm.
HSV-1 gB (COS-1 cells) Gilbert *et al.*, 1994	NE-INM (also ONM, ER, Golgi, and cell surface)	1. gB is targeted to INM in the immature N-glycoform. 2. TM of gB is form aa.#727-795 and consists of three hydrophobic segments. 3. Segment 3 is sufficient to target VSV G protein to nuclear envelope when it replaces the TM of G protein in the fusion. Note that the fusion protein still has the signal peptide from G protein and also localizes to ER, Golgi, and cell surface.
Rat LAP2 (Hela cells) Furukawa *et al.*, 1995	INM (not detected on ER, Golgi, and cell surface)	1. LAP2 has 452 amino acid; type II protein with 409 aa., nucleoplasmic domain; TM is between #410-433. TM: VPMWIKMLLFALVAGFLFLYQAM 2. TM is likely to function as membrane anchor and is not required or sufficient for INM targeting. Domain between #298-370 is essential, but not sufficient for Triton X-100 stable INM targeting. Domain #298-370 possibly functions to bind lamina and thus retain protein in INM.

Table 1: Inner Nuclear Membrane Proteins

Rat gp210 (Mouse Balb/c 3T3 cells) Wozniak and Blobel, 1992	Membrane domain of NPC	1. gp210 is an integral membrane N-glycoprotein. 2. 1886 aa in total; TM-#1806-1818; 58 aa. C-terminal domain. TM: SYQVMFFTFFALLAGTAVTIIAY 3. TM domain of gp210 is sufficient to target CD8-TM fusion to NPC (CD8 is a plasma membrane protein with an internal signal/stop transfer sequence; the TM and C-terminal domain of CD8 were deleted in the fusion). 4. The 58 aa. C-terminal domain of gp210 is a weak, but sufficient NPC targeting signal when fused after the TM domain of CD8 (the CD8 C-terminal domain was replaced).
Otefin (Drosophilia) Harel *et al.*, 1989 Paden *et al.*, 1990	INM	1. Targeting signal is unknown.
Turkey p18 (erythrocytes) Simos *et al.*, 1996	INM	1. p18 is a component of a complex consisting of LBR, lamins, LBR kinase, p34, and p18. 2. Targeting sequence is unknown.
Rat LAPIC Senior and Gerace, 1988 Martin *et al.*, 1995	INM	1. 506 aa in total; TM is between #311-333; type II protein with N-terminal nucleoplasmic domain. 2. Targeting signal is unknown.

Table 2:
Summary of Hydrophobic Sequences in Baculovirus ODV Envelope Proteins (Structural and Functional Cognates of 23AA of ODV-E66)

Protein	Location	Sequence
Autographa californica multinucleocapsid nuclear polyhedrosis virus (AcMNPV)		
ODV-E66 Hong *et al.*, 1994, 1996	N-terminus (23 aa)	MSIVLIIVIVVIFLICFLYLSNS
ODV-E25 Ayres *et al.*, 1994 Hong *et al.*, 1996	N-terminus (24 aa)	MWGIVLLIVLLILFYLYWTNALNF
ODV-E56 Theilmann *et al.*, 1996	Internal (19 aa)	IILLIGAVLFLGLIFYFIY
ODV-E18 Braunagel *et al.*, 1996b	Internal (19 aa)	MFLTILAVVVIIALIIIFV
Orgyia pseudotasugata multinucleocapsid nuclear polyhedrosis virus (OpMNPV)		
ODV-E25 Russell and Rohrmann, 1993	N-terminus (19 aa)	MWGALILLILLVFLFYLWY
ODV-E56 Theilmann *et al.*, 1996	Internal (19 aa)	LIWLIGAVLFLALVVYLIY

Table 2, Continued

Bombyx mori multinucleocapsid nuclear polyhedrosis virus (*Bm*MNPV)

ODV-E66 Maeda *et al.*, 1996 * (GenBank Access #L33180)	N-terminus (21 aa)	MSTVLIIVVVVIFLICFWCLL
ODV-E25 Maeda *et al.*, 1996 * (GenBank Access #L33180)	N-terminus (18 aa)	MWKIVLLIVLLVLIYLYW
ODV-E56 Theilmann *et al.*, 1996	Internal (19 aa)	IIMMIGAVLFLGLILYFIY
ODV-E18 Braunagel *et al.*, 1996b	Internal (19 aa)	MFLTILAVVVIIALIIMFV

Helicoverpa zea singlenucleocapsid nuclear polyhedrosis virus (*Hz*SNPV)

ODV-E56 Theilmann *et al.*, 1996	Internal (19 aa)	IIVVLGIVLLIIFIGYIVI
ODV-E18 Braunagel *et al.*, 1996b	Internal (19 aa)	MLMTILIALVIIILLIMLF

Choristoneura fumiferana multinucleocapsid nuclear polyhedrosis virus (*Cf*MNPV)

ODV-E56 Theilmann *et al.*, 1996	Internal (19 aa)	LIWLIGAVLFLGLIIYLIY

Table 2, Continued

Cydia pomonella granulosis virus (CpGV)

ODV-E56	Internal (19 aa)	ILLVIGGILLLTFIGFVIF

Theilmann *et al.*, 1996

* This is not published and the sequence was obtained from GenBank accession #L33180.

EXAMPLES

The following examples illustrate selected modes for carrying our the claimed invention and are not to be construed as limiting the specification and claims in any way. These examples are provided so as to enable one of ordinary skill in the art to make and use the invention. These examples are not intended to limit the scope of what the inventors regard as the invention. Efforts have been made to ensure accuracy with respect to numbers used to characterize the conditions; however, some experimental errors and deviations may be present.

EXAMPLE 1

MATERIALS AND METHODS USED FOR THIS INVENTION

Insect Cells and Viruses

Wild type AcMNPV (E2 strain), β-gal mutant viruses, and viruses overexpressing 23GFP, 23URF-13 or ODV-E66 were used to infect *Spodoptera frugiperda* (Sf9) cells at a multiplicity of infection (moi.) of 20 (Summers and Smith, 1987). One of the β-gal mutant virus, 23β-gal (Fig. 2, Construct #4) was also used to infect *Trichoplusia ni* (TN368) cells (moi. = 20). Time zero was defined as the time when cells were inoculated with the virus. The virus inoculum was removed after 1 hr absorption.

Virus Purification, Fractionation, ODV-E66 Band Isolation, and N-Terminal Amino Acid Sequencing

To purify the virus, media and cells were collected 5 days postinfection and centrifuged at 9000 rpm in a Beckman JA14 rotor. BV was purified from the supernatant essentially as described by Summers and Smith (1987). The supernatant was centrifuged at 100,000 x *g*, 4°C (Beckman Type 35, 30,000 rpm) to pellet the virus. Virus derived from 500 ml of cell supernatant was resuspended in 1 ml 0.1x TE (TE = 10 mM Tris, 1.0 mM EDTA, pH 7.4) and overlaid onto an 11 ml 25-56% sucrose gradient in 0.1x TE. Gradients were centrifuged at 100,000 x *g* for 90 min at 4°C (Beckman SW41, 24,000 rpm). The

virus band was removed, diluted 1:4 in 0.1x TE, and re-pelleted by centrifugation at 100,000 x *g* for 30 min at 4°C (SW41, 24,000 rpm). The virus was resuspended in 0.1x TE (approximately 0.5 ml per liter of cell supernatant) and stored at 4°C.

Viral occlusions were isolated from infected cells by the method of Whitt and Manning (1987) with some modifications. Cells harvested above (approximately 5 x 10^9 cells) were resuspended in 15 ml of 0.2% Triton X-100 and lysed by sonication on ice (6 X 30 sec, output 5, duty cycle 50%; Model W-375 sonicator; Heat Systems Ultrasonics, Inc.). The sonicates were brought to 80 ml with 0.2% Triton X-100 and the lysate was laid over four 10 ml 30% sucrose (w/v)/0.2% Triton X-100 pads. Viral occlusions were pelleted by centrifugation at 9000 rpm for 20 min in Beckman JA-21 rotor. Each pellet of partially purified viral occlusions was resuspended in 3-4 ml of 0.2% Triton X-100, laid onto a 35 ml 35-60% (wt/wt) sucrose gradient made in water, and centrifuged at 100,000 x*g* for 30 min at 4°C (Beckman SW28, 24,000 rpm). The viral occlusion band was removed and washed twice by diluting in H_2O and low speed centrifugation. The viral occlusions were resuspended in a small volume of H_2O and stored at 4°C. Protein concentration was determined by absorbance at 550 nm (extinction coefficient = 0.28; Summers and Smith, 1987).

ODV was purified from viral occlusions by the method of Tweeten *et al.*, (1980) with some modifications. ODV was liberated from viral occlusions (40 mg/ml) by incubating at 37°C for 2 hr in diluted alkaline solution (0.1 M $NaCO_2$, 0.5 M NaCl, pH 10.9). Approximately 1 ml of alkali-treated viral occlusions was laid onto an 11 ml 25-56% (w/v) sucrose/10 mM Tris (pH 7.5) gradient and centrifuged at 50,000 x *g* for 30 min at 4°C (SW41, 17,500 rpm). Multiple ODV bands were removed, washed by diluting in 10 mM Tris (pH 8.5), pelleted, and finally resuspended with 10 mM Tris (pH 8.5).

Purified viruses were fractionated into envelope and nucleocapsid preparations using modifications of the protocol of Whitt and Manning (1987). In a 250 µl reaction, 200 µg of BV or ODV were incubated in 1% NP40, 10 mM Tris, pH 8.5 at room temperature for 30 min with

gentle agitation. The solution was then laid onto a 4 ml 30-70% (wt/vol) glycerol/10 mM Tris (pH 8.5) gradient and centrifuged at 150,000 x *g* for 60 min at 4°C (Beckman SW60, 34,000 rpm). The envelope fraction was then recovered from the top of the gradient, while the nucleocapsids formed a band approximately 2/3 into the gradient. Both fractions were dialyzed against 10 mM Tris (pH 7.4) overnight at 4°C. All fractions were concentrated using an Amicon Centricon-3 microconcentrator.

For N-terminal amino acid sequencing, purified viral envelope and nucleocapsid fractions were denatured in disruption buffer (2% SDS, 1% β-mercaptoethanol, 25 mM Tris, 7% glycerol, and 0.1% bromophenol blue, pH 6.8) and proteins were separated on a 3% stacking/12.5% separating gel that was pre-run for 30 min with 200 mM thioglycolic acid added to the upper buffer. The separated proteins were electrophoretically transferred to the PVDF membrane (Pro-Blott; Applied Biosystems). Following transfer, the membrane was stained (45% methanol, 5% acetic acid, 0.1% Coomassie blue R250), destained (45% methanol, 5% acetic acid), and washed with water. Two protein bands were excised and N-terminal sequenced. The sequence for a 66 kDa band unique to the ODV envelope was determined to be MSIVLIIVI (Biotechnology Instrumentation Facility, Univ. of Calif., Riverside, CA). A degenerate oligonucleotide was predicted from these amino acids and used to screen AcMNPV genomic DNA digested with *PstI* by standard Southern-blot techniques (Sambrook *et al.*, 1989).

Plasmid cloning and DNA sequencing

Plasmid cloning was described by Sambrook *et al.*, (1989). Double-stranded DNA sequencing was done according to Sequenase 2.0 protocols (USB) using α-$^{[35]}$S-dATP (DuPont NEN Research Products). Oligonucleotides were synthesized on a Model 391 PCR-MATE DNA Synthesizer (Applied Biosystems Inc.). DNA was sequenced on both strands and each nucleotide was sequenced an average of six times. The nucleotide and predicted amino acid sequences were analyzed using the programs of genetics computer group (Devereux *et al.*, 1984;

UWGCG, version 8.0; Madison, WI). The completed gene sequence has been deposited with the GenBank Data Library under Accession No. M96360.

Heterologous protein expression and antibody production

The region from amino acid 24 to 705 of the ODV-E66 ORF was amplified by polymerase chain reaction (PCR; 1 min at 94°C, 1 min at 37°C, and 3 min at 72°C with 5 sec extension after each cycle, 25 cycles) using two primers (forward: 5'-AAAAAAGCTTGTTAACAATAAAAATGATGCC-3' and reverse: 5'-AAAAGGATCCTTACACAA TTTCAAA-3') and Taq polymerase (Promega).

The resultant PCR fragment was extracted with phenol/ chloroform, precipitated with ethanol, digested with *HindIII* and *BamHI*, separated on a 1% agarose gel, purified (GeneClean II, BIOL101, Inc.), and ligated into the *HindIII* and *BamHI* sites of pUC18 to give rise to PCR-pUC18. The FLAG epitope (amino acid sequence: DYKDDDDK;VWR Scientific) was cloned in frame at the N-terminus of the amplified region (amino acids 24-705) to allow affinity purification of the fusion protein when necessary. A linker containing *HindIII* sites on both ends (underlined below) was cloned into the *HindIII* site of the PCR-pUC18 clone. The forward sequence of the linker is shown below: 5'-AAGCTTCATATGGACTACAAAGACGACGACGACAAGCTT-3'. Immediately following the *HindIII* site on the left, there is an *NdeI* site (italic) containing an ATG in frame with the ODV-E66 ORF. DNA sequencing confirmed that the final construct encodes the following N-terminal amino acid sequence: *MDYKDDDDK*LVN (amino acid 24 of ODV-E66 ORF is underlined). The N-terminal eight amino acids (italic) encode the FLAG epitope. Finally, the FLAG-E66 ORF was cloned into the NdeI and *BamHI* sites of pET-11a (Novagen, Inc.) and expressed. The recombinant protein was found in the inclusion fraction of *E. coli*. Bacterial inclusion fraction was prepared as described by the manufacturer (Novagen).

Three New Zealand White female rabbits were injected intramuscularly with approximately 500 μg of the inclusion fraction

in Freund's complete adjuvant (Pierce). The rabbits were then given two additional injections in Freund's incomplete (Pierce) at monthly intervals. One week after the final injection, sera were collected by exsanguination of the rabbits. To eliminate the background reaction, the polyclonal antiserum was preadsorbed with uninfected Sf9 cells. Uninfected Sf9 cells were pelleted, resuspended in 1 X BLOTTO (1% non-fat dry milk, 50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 0.02% NaN_3, pH 7.4), and sonicated until all cells were lysed. ODV-E66 antiserum or preimmune serum was added (1:500) and allowed to bind overnight at 4°C. The antibody was then clarified by centrifugation for 20 min at 15,000 rpm in a Beckman JA-21 rotor. Preadsorbed polyclonal antiserum and preimmune serum were stored at -20°C until use.

To prepare a better antibody which does not require preabsorption to be monospecific for ODV-E66, induced crude *E. coli* lysate was separated on a 10% SDS-PAGE gel. Gel strips were cut from both sides of the gel, stained with Coomassie blue (Sambrook *et al.*, 1989), aligned with an unstained part of the same gel, and the band containing the expressed protein was excised. Then, the unstained gel slice was forced through a gauge 22 needle before letting protein diffuse out of gel by shaking in 50 ml TBS (50 mM Tris, 150 mM NaCl, pH 7.4) overnight at 4°C. Diffused protein was concentrated in a Centriprep-30 (Amicon). The protein concentration was determined by Bradford assay (Bradford, 1976). Two New Zealand White female rabbits were injected subcutaneously four times with a total of 220 ug of protein per rabbit in RIBI adjuvant (RIBI ImmunoChem Research) as described by Harlow and Lane (1988). Nine days after the final injection, sera were collected by exsanguination. Preimmune sera were collected before the first injection. Western-blot analyses showed that the antiserum was monospecific for ODV-E66.

Immunoelectron microscopy (IEM)

To prepare samples for IEM, infected Sf9 or TN368 cells, transfected Sf9 cells, and freshly purified viruses were pelleted and

sequentially fixed: 1% paraformaldehyde, 0.5% glutaraldehyde, 0.05 M sodium cacodylate, pH 7.1, 10 min at 4°C; 2% paraformaldehyde, 2.5% glutaraldehyde, 0.05 M sodium cacodylate, pH 7.1, 30 min at 4°C (all EM chemicals except gold-conjugates were purchased from Electron Microscopy Science). Fixed pellets were washed (0.05 M sodium cacodylate, pH 7.1; 3 X 10 min at 4°C), postfixed (1% osmium tetraoxide, 0.05 M sodium cacodylate, pH 7.1; 30 min at room temperature), and washed again. After fixation, samples were dehydrated and infiltrated following a protocol described by Vandenbosch (1991). Briefly, the pellets were dehydrated at 4°C with 30% ethanol for 30 min and then at -20°C with the following graded ethanol series: 50%; 70%; 90%; 100% (30 min/step). Samples were infiltrated with 50%, 75%, 100% LR White/ethanol series -20°C (1 hr per step). Samples were then infiltrated with 100% LR White at -20°C overnight, followed by another 8 hr infiltration. The resin was polymerized in gelatin capsules heated to 50°C for approximately 48 hr.

Ultrathin sections were cut on a Reichert-Jung ultracut E microtome and collected on nickel grids (Electron Microscopy Sciences), etched (0.56 M sodium meta-periodate, 1 hr; Sigma), washed (H_2O, 2 min), incubated in 0.1 N HCl (10 min), and washed (H_2O, 4 X 2 min). The sections were blocked with TTBS-BSA (1% BSA, 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) for 15 min, reacted with primary antibody (1:500 E66 preabsorbed antiserum, 1:1250 β-gal mouse ascitic fluid, or 1:1500 GFP antiserum; 16 hr at 4°C), and washed (50 mM Tris, 150 mM NaCl, pH 7.4; 4 X 2 min). The bound primary antibody was detected using anti-rabbit IgG 15 nm or 30 nm gold conjugate or anti-mouse IgG 30 nm conjugate (Amersham). The sections were stained with uranyl acetate (Bozzola and Russell, 1992) and lead citrate (Venable and Coggeshall, 1965), and visualized with a Zeiss 10C transmission electron microscope (Electron Microscopy Center, Department of Biology, Texas A&M University, College Station, TX).

Deletion and site-directed mutagenesis

To construct the 125β-gal plasmid, the KpnI-KpnI fragment (28.1-28.2 m.u., Fig. 1a, Hong *et al.*, 1994) within the PstI-F region (23.5-30.1 m.u.; cloned into pUC19) was replaced with a KpnI linker, 5'-GGTACCAAGGATCCTTGGTACC-3' (BamHI site is underlined). A 3.1 kb BamHI fragment containing the *lacZ* gene from plasmid pAc360-β gal (Luckow and Summers, 1989) was inserted into the BamHI site so that the region encoding N-terminal 125 amino acids of ODV-E66 was fused in frame with the *lacZ* gene.

To introduce partial deletions within the N-terminal 125 amino acids of 125β-gal, the EcoRI-K region (25.0-29.2 m.u., Fig. 1a, Hong *et al.*, 1994) containing wild type *ODV-E66* was cloned into the EcoRI site of pUC19 as the template for mutagenesis using transformer site-directed mutagenesis kit (ClonTech; See Construct #2, Fig. 2, "125β-gal"). Both deletion and site-directed mutagenesis were done as instructed by the manufacturer. All mutants were confirmed by DNA sequencing (Sequenase 2.0; USB).

To obtain the construct Δ2-23β-gal (See Construct #3, Fig. 2), amino acids #2-23 were deleted using the deletion oligonucleotide 5'-GCAACATTCGACATGAGCAATAATAAAAATGATGCC-3' and the selection oligonucleotide 5'-CTCTAGAGGAACCCCGGGAACCGAGCTCG-3'. After the introduction of deletion, the KpnI-KpnI fragment (28.1-28.2 m.u.) was replaced with the 3.1 kb KpnI fragment containing the *LacZ* gene from 125β-gal. Construct #3 is the same as construct #2 except that the amino acids #2-23 (22 amino acids total) are deleted. The fusion protein Δ2-23β-gal is cytoplasmic.

To obtain the construct 23β-gal (See Construct #4, Fig. 2), nucleotide sequence encoding amino acids #24-25 were mutated to a KpnI site using the selection oligonucleotide 5'-CTCTAGAGGAACCCCGGG AACCGAGCTCG-3' and the site-mutation oligonucleotide 5'-CCTATCAAATAGC GGTACCAAAAATG ATGCCAAT-3' (KpnI site is underlined). The region from the new KpnI site at amino acids #24-25 to the KpnI site at 28.2 m.u. was replaced with the 3.1 kb KpnI fragment containing the *LacZ* gene from 125β-gal.

Cloning strategy for overexpression of 23GFP

The N-terminal 23 amino acids of ODV-E66 (**MSIVLIIVIVVIFLICFLYLSNS**) was amplified by PCR (30 sec at 94°C, 60 sec at 42°C, 90 sec at 72°C, 25 cycles) using the following oligonucleotides: 5'-TTTTTTAAGCTTATGTCTATCGTATTG-3' (HindIII site is underlined) and 5'-TTTTTTGGATCCTTGCTA TTTGATAGGTA-3' (BamHI site is underlined). The PCR product was extracted with chloroform, precipitated with ethanol, digested with HindIII and BamHI, gel purified, and cloned into the HindIII and BamHI sites of pUC19. Amplified region was verified by DNA sequencing. The BamHI/EcoRI fragment from pGFP vector (ClonTech) which contains the complete GFP open reading frame (ORF) was cloned into the BamHI/EcoRI sites of the pUC19 PCR clone so that N-terminal 23 amino acids of ODV-E66 were fused in frame with GFP (pUC19-23GFP).

The clone pVL1393-23GFP (See Construct #6, Fig. 2) was generated to overexpress 23GFP in insect cells. The HindIII site in pUC19-23GFP was filled by Klenow and the HindIII-EcoRI fragment was cloned into the SmaI/EcoRI sites of transfer vector pVL1393 (Webb and Summers, 1990). In this construct, expression of 23GFP was controlled by the polyhedrin promoter. Infected cells have an occlusion minus phenotype.

The 23GFP ORF was then cloned into the transfer vector pAcUW21 (PharMingen). The region from the BamHI site in the multiple cloning region of pVL1393-23GFP to the EcoRI site at the 3'-end was cloned into the BglII/EcoRI sites of pAcUW21 (ClonTech). In this construct (pAcUW21-23GFP; See Construct #5, Fig. 2) expression of 23GFP was under the control of the p10 promoter. Infected cells have an occlusion positive phenotype.

A recombinant virus containing GFP under the control of the polyhedrin promoter was provided by Dr. Christian Oker-Blom (VTT Biotechnology, Finland; Laukkanen *et al.*, 1996; Oker-Blom *et al.*, 1996) and was used as a control for immunofluorescence and immunogold labeling.

Cloning strategy for overexpression of ODV-E66

To express ODV-E66 at high levels under the polyhedrin promoter and in the polyhedrin locus, *ODV-E66* was amplified by PCR (1 min at 94°C, 1 min at 37°C, and 3 min at 72°C with 5 sec extension after each cycle, 25 cycles) using the following oligonucleotides: 5'-TTTTTGGATCCACCATGTCTATCGTATTG-3' (BamHI site underlined) and 5'-TTTTTCTGCAGTTA*CTTGTCGTCGTCGTCTTTGTAGTC*CACAATTTCAAAAAT-3 ' (PstI site underlined). The second oligonucleotide contains the "FLAG" epitope (italic; amino acid sequence: DYKDDDDK; VWR Scientific) immediately upstream of the stop codon. The PCR product was cloned into the BamHI and PstI sites of pVL1393 (Webb and Summers, 1990). The resultant construct (pVL1393-E66F; See Construct #7, Fig. 2) was sequenced to verify that PCR did not introduce errors in the nucleotide sequence.

Cloning strategy for overexpression of 23URF13

To overexpress a fusion gene encoding N-terminal 23 amino acids fused in frame with URF13 gene (Dewey *et al.*, 1986) under the control of p10 promoter in the pAcUW21 transfer vector, recombinant PCR was used to amplify and combine the nucleotide sequence encoding 23 amino acids of ODV-E66 with the URF13 gene. N-terminal 23 amino acids of ODV-E66 was amplified by PCR (60 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C, 25 cycles) using the following oligonucleotides: 5'-TTTTCGGGATCCATGTCTATCGTATTG-3' (BamHI underlined) and 5'-TAAGAAAGTAGTGATGCTATTTGATAGGTA-3'. URF13 gene was amplified by PCR (60 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C, 25 cycles) using the Turf-2b plasmid as template (Dewey *et al.*, 1986) and the following oligonucleotides: 5'-TACCTATCAAATAGCATCACTACTTTCTTA-3' and 5'-AAAAAGGAATTCTCACGGTACTTGTAC-3' (EcoRI site underlined). After the first round of PCR for 23 amino acids and the URF13 gene, 1 ul of PCR product from each PCR reaction was used as the templates for a second round of PCR (60 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C, 25 cycles) using two of the oligonucleotides used in the first PCR: 5'-TTTTCGGGATCCATGTCTATCGTATTG-3' (BamHI underlined) and 5'-

AAAAAGGAATTCTCACGGTACTTGTAC-3' (EcoRI site underlined). Product from the second PCR was extracted with chloroform, precipitated with ethanol, digested with BamHI and EcoRI, and cloned into the BglII/EcoRI sites of pAcUW21 vector. Recombinant virus made using this construct (pAcUW21-23URF13; See Construct #8, Fig. 2) produces viral occlusions in infected cells.

Construction of Recombinant Viruses

Recombinant viruses 125β-gal, Δ2-23β-gal, and 23β-gal, were constructed using the corresponding transfer plasmid and *Ac*MNPV viral DNA (Summers and Smith, 1987). pVL1393-23GFP, pAcUW21-23GFP, pVL1393-E66F, and pAcUW21-23URF13 were constructed using the corresponding plasmid constructs and Bsu36I-digested BakPak6 viral DNA (ClonTech). The locus of recombination was verified by Southern analyses and an appropriate probe. Protein expression was confirmed by Western-blot analyses of infected cell extracts using appropriate antibody: β-gal recombinant viruses (mouse anti-β-gal polyclonal antibody at 1:1250; Sigma); 23GFP recombinant viruses (rabbit anti-GFP polyclonal antibody at 1:1500; ClonTech). Southern and Western-blot analyses were done as described by Sambrook *et al.*, (1989).

Fig. 12 is genomic map of *Autographa californica* NPV showing a construct containing dual promoters and which is occlusion positive and contain both 23URF13 and wild type t-URF13 (WT t-URF13). Multiple genes can be inserted at both the polyhedrin and p10 loci. In this manner, recombinant viruses can be constructed that are occlusion positive and contain multiple genes. Genomic map modified and reprinted from Ayres, *et al.*, Virology, 202:586-605 (1994). Construction of recombinants containing multiple genes is routine for the inventors using the techniques described by Belyaev and Roy, Nucleic Acid Research, 21:1219-1223 (1993) and Summers and Smith, Texas Agricultural Experiment Station, Bulletin No. 1555 (1987).

GFP Autofluorescence

Autofluorescence of GFP in cells expressing GFP or 23GFP was observed on a Zeiss Axiophot microscope (Electron Microscopy Center, Texas A&M University) equipped with a filter set for fluorescein isothiocyanate (FITC). Cells were photographed using phase contrast, fluorescence, or double exposure of phase contrast and fluorescence. Exposure time for cells at different time points postinfection was maintained constant and was set for proper exposure of the 72 hr p.i. time point for comparison of fluorescence intensity.

Transient Expression of 23GFP in Uninfected Sf9 Cells

To transiently express 23GFP under the control of the IE1 promoter, the HindIII/EcoRI fragment from 23GFP-pUC19 was filled with Klenow at both ends and cloned into the PmeI site of pIE1HR4 (Jarvis *et al.*, 1996) so that the filled HindIII site was close to the BamHI site in pIE1HR4. This construct was named pIE1HR4-23GFP (See Construct #9, Fig. 2).

Transfection of plasmid DNA into uninfected Sf9 cells was done using the CellFECTIN lipofection reagent as instructed by the manufacturer (Gibco BRL). For each transfection, 2 ug of plasmid DNA and 9 ul of CellFECTIN reagent were used. Cells were recovered at 24 or 48 hr post-transfection for fluorescence observation or EM fixation. The pattern of protein localization was indistinguishable between 24 and 48 hr post-transfection.

Transient expression of 23GFP in mammalian (COS-1) cells

The pUC19-23GFP construct (see Cloning Strategy for Overexpression of 23GFP) was digested with EcoRI, filled with Klenow (Sambrook *et al.*, 1989), Klenow enzyme inactivated by heating at 75°C for 15 min, and digested with HindIII. Then, the resulting HindIII/EcoRI fragment containing 23GFP was cloned into the HindIII/HpaI sites of pCMV Blue vector (PharMingen). In this construct (pCMV-23GFP; See Construct #10, Fig. 2) expression of 23GFP is under the control of

the major immediate early promoter of cytomegalovirus which is active in the COS-1 cells.

COS-1 cells (Gluzman, 1981) were maintained at 37°C in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum. Transfection of plasmid DNA into COS-1 cells was done in DMEM medium without serum and antibiotics using the CellFECTIN lipofection reagent as instructed by the manufacturer (Gibco BRL). For each transfection, 2 ug of plasmid DNA and 15 ul of CellFECTIN reagent were used. After transfection, cells were incubated at 37°C for 24 hr for 23GFP to be expressed, then cells were transferred to 30°C and incubated for another 48 hr for GFP autofluorescence to develop (Ogawa *et al.*, 1995).

EXAMPLE 2
IDENTIFICATION, SEQUENCE, AND ANALYSIS OF ODV-E66 AND THE N-TERMINAL 23 AMINO ACID HYDROPHOBIC DOMAIN

This Examples teaches the identification, sequence determination and analyses of ODV-E66 and the N-terminal 23 amino acid hydrophobic domain.

Baculovirus Expression of AcMNPV ODV-E66 and Overexpression of ODV-E66 Under the Control of the Polyhedrin Gene Promoter in the Polyhedrin Locus

To identify proteins unique to the ODV envelope, ODV and BV were purified and fractionated into envelope and nucleocapsid fractions. A comparison of the structural proteins of ODV, BV, and envelopes revealed a protein of 66 kDa unique to the ODV envelope (Braunagel and Summers, 1994, incorporated herein by reference). N-terminal amino acid sequencing of this protein determined the sequence of: **M S I V L I I V I** . The gene was mapped to the *EcoRI-PstI* region (25.0-30.1 m.u., Fig. 1 of Hong *et al.*, 1994, incorporated herein) of the AcMNPV genome and the nucleotide sequence (2115 nucleotides of open reading frame) of the gene encoding ODV-E66 predicts a protein of 704 amino acids (Fig. 1).

Fig. 1A is a diagram representing the nucleotide and amino acid sequences of the gene encoding ODV-E66. Nucleotide sequences upstream and downstream of ODV-E66 open reading frame are also included. The amino acid sequence of ODV-E66 is shown in single capital letters. The numbers to the left indicate positions of nucleotide and amino acid. The N-terminal 23 amino acid hydrophobic sequence is boxed thereby including nucleotide numbers 122 through 190 (sixty-nine (69) nucleotides). Restriction endonuclease sites (DraI, KpnI, and EcoRI) are underlined. Arrows above the nucleotide sequences (TTAAG and ATAAG) indicate the transcription initiation sites.

The nucleotide sequences and amino acid sequences for the 23AA sequence of this invention are shown below (see Fig. 1B):

```
A T G  T C T  A T C  G T A  T T G  A T T  A T T  G T C
M      S      I      V      L      I      I      V

A T A  G T T  G T A  A T A  T T T  T T A  A T A  T G T
I      V      V      I      F      L      I      C

T T T  T T G  T A C  C T A  T C A  A A T  A G C
F      L      Y      L      S      N      S
```

The 23 AA sequence has the following amino acid sequence:

M S I V L I I V I V V I F L I C F L Y L S N S.

Studies of the gene encoding ODV-E66 demonstrated that it is a baculovirus late gene with transcription initiating from conserved TAAG motifs (Fig. 1A, arrows; For complete description of genetic analysis including Northern-blot, primer extension, 5' and 3' S1 nuclease analysis see Hong *et al.*, 1994).

Fig. 2 is an outline summarizing the different constructs used in this invention and includes the construct name, gene promoter and locus, occlusion production, and protein location in cells. Numbers above the structures indicate locations of amino acids.

In Construct #1, ODV-E66, the ODV-E66 expression is controlled by its native promoter in the native locus in wild type virus and infected cells produce viral occlusions.

Fig. 3A, 3B and 3C are photographs showing immunogold labeling of purified ODV and wild type AcMNPV infected Sf9 cells at 48 hr p.i. Rabbit ODV-E66 antiserum and anti-rabbit IgG 15 nm gold-conjugate were used. Fig. 3A: Labeling of the viral envelope of purified ODV (arrowhead). Fig. 3B: Labeling of the intranuclear viral-induced microvesicles. Note the microvesicles are surrounded by fibrillar structures and viral occlusions. Fig. 3C: Labeling of the viral

envelope of ODV being occluded into a viral occlusion (arrowhead). The following abbreviations are used in this figure: m, microvesicles; f, fibrillar structure; oc, viral occlusion.

Studies of the protein ODV-E66 show that it is a structural protein of the ODV envelope (Fig. 3A, arrowheads; Hong *et al.*, 1994). During viral assembly, ODV-E66 locates to viral induced intranuclear microvesicles (Fig. 3B, m) and the ODV envelope that is maturing into the occluded form (Fig. 3C, arrowheads; Hong *et al.*, 1994).

A recombinant virus was generated that places an additional copy of the gene encoding ODV-E66 into the baculovirus genome under the control of the polyhedrin promoter (Fig. 2, Construct #7). In construct #7, an extra copy of ODV-E66 gene replaces the polyhedrin gene in the polyhedrin locus in Construct #7. In this construct, (a) expression of the extra copy of ODV-E66 gene is under the control of polyhedrin promoter, (b) infected cells did not produce viral occlusions due to the absence of a functional polyhedrin gene, and (c) the ODV-E66 gene in the native locus is intact.

Figs. 4A, 4B, and 4C are photographs showing immunogold labeling of Sf9 cells infected with pVL1393-E66F at 48 hr p.i. Rabbit ODV-E66 antiserum and anti-rabbit IgG 15 nm gold-conjugate were used. Fig. 4A: Labeling in the intranuclear microvesicles and cytoplasmic membranes close to the nuclear envelope (dual arrow). Fig. 4B: Labeling of the cytoplasmic membranes (dual arrow). Fig. 4C: Labeling of the outer (arrowhead) and inner (arrow) nuclear membranes. The following abbreviations are used in this figure: m, microvesicles; n, nucleus; c, cytoplasm.

Immunoelectron microscopy of the localization of ODV-E66 in this recombinant virus shows the same protein localization as wild type AcMNPV, however the amount of ODV-E66 is increased significantly (Fig. 4; Hong *et al.*, 1996). By increasing the amount of ODV-E66 we could detect the same labeled structures as wild type, but additionally can visualize cellular locations of the protein that were indicated by wild type virus, but were observed at low levels. These locations include: ODV envelope (Fig. 4A and 4C), microvesicles

and other intranuclear membranes (Fig. 4A, 4C; m), membranes closely associated with the nucleus (Fig. 4A, 4B, dual arrow), the nuclear envelope, including the inner and outer nuclear membrane (4C, INM, arrow; ONM, arrowhead).

Overexpression of ODV-E66 by this recombinant virus confirms that ODV-E66 locates to the nuclear membrane, an observation that was indicated with wild type virus, but difficult to confirm due to decreased levels of proteins. Additionally, pVL1393-E66F generated virus confirms that when ODV-E66 (or derived fusion) is placed in the baculovirus genome at an alternative locus (*i.e.*, polyhedrin locus) and under the control of an alternate promoter (*i.e.*, polyhedrin or p10 or other classes of viral gene promoters), it locates to the same cellular organelles and structures as it would locate in wild type baculovirus infected cells.

Analysis of the N-terminal 23 Amino Acids of ODV-E66, Development of Recombinant Viruses Containing Fusion Proteins, and Localization of Fusion Proteins During Viral Infection

Beta-Galactosidase Protein (β-gal) as a Marker

A mutant virus was constructed in which a fusion gene encoding the N-terminal 125 amino acids of ODV-E66 was fused in frame with β-galactosidase (β-gal; Fig. 2; 125β-gal, Construct #2) and replaced *ODV-E66* in the native locus.

Construct #2 shows that the ODV-E66 gene, in the native locus, is replaced by a mutant gene 125β-gal (in frame fusion of N-terminal 125 amino acids of ODV-E66 with β-galactosidase of *E. coli*) whose expression is still controlled by the native ODV-E66 promoter.

Because β-gal is cytoplasmic when expressed in infected Sf9 and Sf21 cells (Jarvis *et al.*, 1991; Hershberger *et al.*, 1994), it was expected that the fusion protein would also locate to the cytoplasm. However, immunofluorescence and immunogold labeling showed that the 125β-gal fusion protein localized into the nucleus and to viral induced intranuclear microvesicles and ODV envelope (Hong, 1995). The conclusion was that the N-terminal 125 amino acids of ODV-E66 must contain the requisite signal(s) necessary to direct β-gal or

potentially other proteins into the nucleus and intranuclear membranes.

Using computer-assisted analysis, an N-terminal hydrophobic region of 23 amino acids with a general hydrophobic character similar to the signal/anchor sequences in many membrane proteins (von Heijne, 1990) was identified:

M S I V L I I V I V V I F L I C F L Y L S N S

Several additional constructs were made which included fusion mutants with or without the N-terminal hydrophobic domain (see Fig. 2). After cloning the gene encoding each mutant protein, a recombinant virus was produced and expression of each gene, under a variety of baculovirus promoters, was studied. These recombinant gene constructs were then inserted at various loci within the baculovirus genome (See Fig. 2).

When the hydrophobic 23 amino acid sequence was fused in frame with the normally cytosolic protein β-galactosidase (23β-gal; Fig. 2, Construct #4), the fusion protein was transported into the nucleus and this was initially confirmed using fluorescent confocal microscopy (Hong, 1995). In construct #4, the ODV-E66 gene, in the native locus, is replaced by a mutant gene 23β-gal (in frame fusion of N-terminal 23 amino acids of ODV-E66 with β-galactosidase of *E. coli*) whose expression is still controlled by the native ODV-E66 promoter.

Figs. 5A and 5B are photographs showing immunogold labeling of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (TN368) cells infected with 23β-gal virus at 48 hr p.i. For these figures, mouse β-gal antiserum and anti-mouse IgG 30 nm gold-conjugate were used. Fig. 5A: 23β-gal fusion protein was localized to the intranuclear microvesicles (arrowhead) and ODV envelope (arrow) in infected Sf9 cells. Fig. 5B: 23β-gal fusion protein was localized to the intranuclear microvesicles (arrowhead) and ODV envelope (arrow) in infected TN368 cells. The following abbreviations are used in this figure: m, microvesicles; n, nucleus; c, cytoplasm.

Immunoelectron microscopy of 23β-gal infected Sf9 cells showed that the N-terminal 23 amino acids of ODV-E66 were sufficient to direct the fusion protein 23β-gal to the viral induced intranuclear microvesicles and ODV envelope (Fig. 5A). These results are not restricted to Sf9 cells, and these results were confirmed using another insect cell line, *Trichoplusia ni* (TN368; Fig. 5B).

Green Fluorescent Protein (GFP) as a Marker

Green fluorescent protein (GFP) is a small protein that normally resides throughout the cells, including the cytoplasm and nucleus and is not normally membrane associated. It is known that if wild type GFP is expressed in baculovirus under the control of the polyhedrin promoter that it also uniformly distributes throughout the cell (Hong *et al.*, 1996). GFP was chosen as an additional marker protein for several reasons:

(a) Because it normally is not excluded from the nucleus, the 23GFP fusion should also indicate specific controls or in the presence of specific localizations factors, for both the cytoplasmic and nuclear compartments; and

(b) The autofluorescence of GFP should allow one to visually track and locate the fusion protein movement after viral infection.

Thus, GFP should provide another example and an overview of specific localization and transport with a high degree of sensitivity and specificity for detection.

Two recombinant viruses were generated which contained the N-terminal 23 amino acids of ODV-E66 fused in frame with GFP (23GFP; Fig. 2, Constructs #5-#6). In construct #5, a fusion gene of N-terminal 23 amino acids with green fluorescent protein (23GFP) was placed under the control of p10 promoter and inserted into the polyhedrin locus as an extra copy of transcriptional unit. The polyhedrin and p10 genes in their native loci are still intact, thus infected cells still produced viral occlusions. In construct #6, the 23GFP fusion gene replaced the polyhedrin gene in the polyhedrin locus. Expression of 23GFP was under the control of polyhedrin

promoter. Infected cells did not produce viral occlusions due to the absence of a functional polyhedrin gene.

Cells infected by pAcUW21-23GFP (p10 promoter) or pVL1393-23GFP (polyhedrin promoter) were occlusion-positive or occlusion-negative, respectively. Cells infected by pAcUW21-23GFP produced normal amounts of viral occlusions and occluded virus as determined by immunogold labeling (Hong *et al.*, 1996). The pattern of label of 23GFP fusion protein location was essentially the same for the occlusion positive and occlusion negative mutants. Because of the additional complexity of occlusion formation within the nucleus, the data using the occlusion-negative mutant has been chosen for the representative observation of GFP localization (Fig. 6).

Figs. 6A-6I are photographs showing localization of 23GFP in pVL1393-23GFP infected Sf9 cells. Fig. 6A: 48 h p.i., GFP autofluorescence. Fig. 6B: 48 h p.i., GFP autofluorescence and phase contrast. Arrows in A and B point to the fluorescence at the periphery of the nucleus. Fig. 6C: 72 h p.i., GFP autofluorescence. Fig. 6D: 72 h p.i., GFP autofluorescence and phase contrast. Arrowheads in C and D point to the fluorescence in the interior of the nucleus. Figs. 6E, 6F, 6G, 6H, 6I: Immunogold labeling of Sf9 cells infected with pVL1393-23GFP at 48 hr p.i. using rabbit GFP antiserum and anti-rabbit IgG 30 nm gold-conjugate. Fig. 6E: Note the labeling of cytoplasmic membranes at the periphery of the nucleus and foci of intranuclear microvesicles. Fig. 6F: Labeling of cytoplasmic membranes (dual arrow), outer (arrow) and inner (arrowhead) nuclear membranes. Fig. 6G: Labeling of large cytoplasmic vesicles (open-arrow). Fig. 6H: Labeling of outer (arrow) and inner (arrowhead) nuclear membranes. Fig. 6I: Labeling of the ODV envelope (arrow). The following abbreviations are used in these figures: c, cytoplasm; n, nucleus; v, virogenic stroma; m, microvesicles.

Immunoelectron microscopy showed that 23GFP located to the same cellular membranes as the 125β-gal and 23β-gal fusion proteins and wild type ODV-E66 (Fig. 6). 23GFP initially locates to regions

closely associated with the nuclear envelope (Fig. 6A-6B; arrow) and as infection progresses, 23GFP moves into the nucleoplasm as discrete foci of microvesicles (Fig. 6C-6D; arrowhead). Immunogold analysis shows that the 23GFP fusion protein locates to the same cellular membranes as wild type ODV-E66. These membranes include: Cytoplasmic membranes juxtaposed to the periphery of the nucleus (Fig. 6E, 6F; dual arrows; 6G, open arrows); Inner and Outer nuclear membranes of the nuclear envelope (Fig. 6F-6H; INM, arrowheads, ONM, arrows); Intranuclear foci of microvesicles (Fig. 6E, m); and, ODV envelope (Fig. 6I; arrows) and other intranuclear membranes.

The data demonstrated that proteins normally cytosolic (β-gal) or freely dispersed between the nucleus and cytoplasm (GFP) are specifically trafficked into defined membranes when fused to the 23 hydrophobic amino acid sequence of ODV-E66.

Plasma Membrane Protein URF13 as a Marker

To confirm that the 23 amino acid sequence was also capable of directing a protein that normally locates to the plasma membrane, the plasma membrane protein URF13 was chosen for additional study. The N-terminal 23 amino acid sequence was fused to wild type URF13 (23URF13; Fig. 2, Construct #8) and the recombinant virus generated. In construct #8, a fusion gene of N-terminal 23 amino acids with URF13 gene was placed under the control of p10 promoter and inserted into the polyhedrin locus as an extra copy of transcriptional unit. In this construct, the polyhedrin and p10 genes in their native loci are still intact, thus infected cells still produce viral occlusions.

Figs. 7A and 7B are photographs showing immunogold labeling of Sf9 cells infected with pAcUW21-23URF13 virus at 48 hr p.i. Rabbit URF13 antiserum and anti-rabbit IgG 30 nm gold-conjugate were used in these experiments. Fig. 7A: Labeling of the ODV envelope (arrow) and intranuclear vesicles (open arrow). Fig. 7B: Labeling of cytoplasmic membranes (arrowhead). The following abbreviations are used in this figure: c, cytoplasm; n, nucleus.

The data showed that 23URF3 located to the same structures as the β-gal and GFP fusion proteins, *i.e.*, the ODV envelope (Fig. 7A, arrow), intranuclear membranes (Fig. 7A, open arrow) and membranes closely associated with the nuclear envelope (Fig. 7B, arrowhead).

Thus, three marker proteins that normally locate to three different regions of the cell were used in this experiment

(1) β-gal is normally cytosolic;

(2) GFP is normally both cytosolic and nuclear; and

(3) URF13 is usually targeted to the plasma membrane.

This data demonstrates that when each of these three different proteins was fused to the N-terminal 23 amino acid sequence of ODV-E66, it was targeted to different cellular locations than their wild type counterparts. Specifically these locations were: cytoplasmic membranes juxtaposed to the nuclear envelope, both the outer and inner nuclear membranes of the nuclear envelope, foci of viral induced intranuclear microvesicles and the ODV viral envelope. These examples clearly showed that in baculovirus infected cells, the 23 amino acid sequence specifically located a protein to distinct membrane structures.

Localization of the Fusion Proteins in Uninfected Insect Cells and Mammalian Cells --- Demonstrated Use of Alternate Promoters, and Potential Cell Cycle Regulation of Protein Location

To determine where this 23 amino acid sequence targets a protein in uninfected cells would more specifically address the role of the 23 amino acids and eliminate the possible role of other viral encoded proteins for the specific localization of the fusion proteins.

Thus, these experiments were performed in both uninfected insect (Sf9) cells and uninfected mammalian (COS-1) cells. To perform these experiments, promoters were chosen that are recognized by the host cell RNA polymerase II. For the insect cells, the promoter from the immediate early gene IE1 of AcMNPV was used (Fig. 2, Construct #9). For the mammalian cells, the promoter of the major immediate early gene of cytomegalovirus was chosen (CMV; Fig. 2, Construct #10).

In construct #9, the 23GFP fusion gene was placed under the control of IE1 promoter in the pIE1HR4 vector for transient expression in uninfected insect cells. This was different from previous constructs #1-#8) which were recombinant viruses expressing proteins in infected cells. For this construct, an IE1 promoter was used (IE1 is an AcMNPV immediate early gene promoter that is active in uninfected insect cells). The fusion gene construct was transfected into Sf9 cells and transient expression was achieved without viral infection. The results showed that in uninfected Sf9 cells, 23GFP protein localized to the nuclear envelope in a cell cycle-dependent manner.

It must be emphasized that a wide variety of promoters from many known genes could have been chosen for this study or any similar experiment with similar results.

Figs. 8A-8H are photographs showing transient transfection of uninfected Sf9 cells with pIE1HR4-23GFP. Figs 8A, 8C, 8E: Fluorescence microscopy at 24 hr post-transfection. Figs. 8B, 8D, 8F: Matched fluorescence and phase contrast image double exposure to fluorescence images in Figs. 8A, 8C, 8E, respectively. Figs. 8G, 8H: Immunogold labeling of binucleated cells at 48 hr post-transfection using rabbit GFP antiserum and anti-rabbit IgG 30 nm gold-conjugate. Fig. 8G: Note small vesicles in the nucleoplasm (arrowhead) and cytoplasmic vesicles juxtaposed to the nuclear envelope (dual arrow in insert). Fig. 8H: Labeling of cytoplasmic vesicles (open arrow), outer and inner nuclear membranes (arrow). Also note the invagination of the inner nuclear membrane into the nucleoplasm (arrowhead). The following abbreviations are used in this figure: c, cytoplasm; n, nucleus; ne, nuclear envelope.

Two general patterns of fluorescence were observed in insect Sf9 cells that were transiently transfected with IE1-23GFP:

(a) Some cells showed diffuse fluorescence indicating that 23GFP was located throughout the cell (Fig. 8C, 8D, 8E, 8F); and

(b) Dividing cells, as determined by the presence of two nuclei in the same cell, showed an enhancement of fluorescence around the

periphery of the nucleus or in the nuclear envelope (Fig. 8A, 8B, 8C, 8D: arrows).

Immunogold labeling of cells harvested at 48 hr post transfection confirmed these observations. Many cells showed labeling throughout the cell, both the cytoplasm and nucleus (data not shown); however, in binucleated cells, 23GFP located to vesiculated cytoplasmic membranes adjacent to the nuclear envelope (Fig. 8H, open arrows), and the outer and inner nuclear membranes (Fig. 8H, arrows). These results indicate that proteins containing the 23 amino acid sequence are differentially regulated to the nuclear membrane during discrete stages of the cell cycle. Germline transformed Sf9 cells have been developed containing the 23GFP fusion protein, and studies using time lapse exposures to closely evaluate protein movement relative to cell cycle are being performed. In mitotic cells, observations show that cytoplasmic membranes vesiculate and locate in close proximity to the nuclear envelope (Fig. 8G, ne, and insert; dual arrows) in a manner very analogous to the type of membrane localization observed in baculovirus infected cells. Thus, scientific discoveries of specific pathways of protein movement in baculovirus infection may extend significantly to currently unknown pathways of protein movement for nuclear membranes and membranes within the nucleus in other cell types that may be regulated by the cell cycle.

Next, a 23GFP fusion product under the control of a promoter which is optimized for mammalian cell expression was constructed (Fig. 2; #10). In construct #10, the 23GFP fusion gene was placed under the control of the major immediate early gene promoter of cytomegalovirus (CMV-IE) in the pCMV vector for transient expression in uninfected mammalian COS-1 cells. The fusion gene construct was transfected into COS-1 cells and transient expression was achieved without viral infection.

In uninfected COS-1 cells, 23GFP protein localized to the nuclear envelope in a cell cycle-dependent manner. Figs. 9A and 9B are photographs showing fluorescence microscopy of mammalian COS-1 cells transiently transfected with pCMV-23GFP. Fig. 9A: Note the ring of

fluorescence (arrowhead) in a cell with two nuclei that is likely in mitosis. Fig. 9B: The ring of fluorescence is not obvious. This cells is possibly not in the mitosis due to the presence of a single nucleus and intact nuclear envelope (arrowhead). Using the pCMV-23GFP construct the results demonstrate that autofluorescence of 23GFP is enriched along the nuclear membranes of bi-nucleated (*ie.*, mitotic) cells (Fig. 9A), while in non-mitotic cells the fluorescence is diffuse (Fig. 9B). This data predicts and supports that the fusion protein containing the ODV-E66 N-terminal 23 amino acids is specifically located to the membranes closely associated with the nucleus, including the inner nuclear membrane, at specific times during the cell cycle. This data also predicts that this sequence functions in a similar manner in both insect and mammalian cells.

EXAMPLE 3

EXPRESSION OF 23 GREEN FLUORESCENT PROTEIN IN PLANT CELLS

This Example presents a method for constructing a plant vector containing the N-terminal 23 hydrophobic amino acids from ODV-E66 and was designed to study the localization of protein (green fluorescent protein) as a result of the 23 amino acid sequence.

To express 23 green fluorescent protein (23GFP) in plant cells, the 23GFP fusion gene amplified by two rounds of PCR in order to be cloned into the plant expression vector pRTL2 (Carrington and Freed, 1990). The first round of PCR includes two PCR reactions (94°C, 30 sec; 42°C, 60 sec; 72 °C, 60 sec; 25 cycles): The first reaction used the pAcUW21-23GFP construct as template and two oligonucleotides (5'-TTTTTTCCATGGCATCTATCGTATTGATT-3' and 5'-GACAAGTGTTGGCCAGGGAACAGGTAGTTTTCC-3'); the second reaction used the pAcUW21-23GFP construct as template and two oligonucleotides (5'-GGAAAACTACCTGTTCCCTGGCCAACACTTGTC-3' and 5'-TTTTTTTCTAGACTATTTGTATAGTTC-3'). The second round of PCR used 1% of each of the first round of PCR reactions and two oligonucleotides (5'-TTTTTTCCATGGCATCTATCGTATTGATT-3' and 5'-TTTTTTTCTAGACTATTTGTATAGTTC-3'; NcoI and XbaI sites underlined). PCR fragment from the second PCR was extracted with chloroform,

precipitated with ethanol, digested with NcoI and XbaI, purified from 1% agarose gel using GeneClean kit (BIOL101), and cloned into the NcoI/XbaI sites of pRTL2 vector. This construct was named pRTL2-23GFP (Fig 2., Construct #11). Thus, in this construct, the 23GFP fusion gene was placed under the control of the 35S promoter in the pRTL2 vector for expression in plant cells. 35S promoter is active in plant cells without viral infection.

23GFP autofluorescence in plant cells can be observed using a FITC filter in a Zeiss Axiophot microscope (Zeiss). Experiments are in progress to determine the location of 23GFP in plant cells.

EXAMPLE 4
EXPRESSION OF A FUSION GENE OF N-TERMINAL 24 AMINO ACID HYDROPHOBIC SEQUENCE OF ODV-E25 WITH GFP IN INSECT SF9 CELLS

ODV-E25 is another ODV envelope protein of baculovirus OpMNPV (Russell and Rohrmann, 1993),and AcMNPV (Ayres *et al.*, 1994), is related to ODV E66, and it is the N-terminal 24 amino acids of AcMNPV ODV-E25 that has been used in this Example. This Example presents a method for constructing a vector containing the 24 N-terminal amino acids from ODV-E25 and forming a fusion product. This construct would be used to identify the presence of a functional cognate in these 24 amino acids.

AcMNPV ODV-E25 was localized to the microvesicles (T. Hong, S. C. Braunagel, and M. D. Summers, unpublished observation) and also contains an uncleaved N-terminal 24 amino acid hydrophobic sequence (Hong *et al.*, 1996). To determine if this 24 amino acid sequence functions similarly to the 23 amino acid sequence of ODV-E66, a fusion construct (24GFP) was made in which the 24 amino acid sequence of ODV-E25 was fused in frame with GFP. The nucleotide sequence encoding the N-terminal 24 amino acids of ODV-E25 was amplified by PCR (94°C, 30 sec; 42°C, 60 sec; 72°C, 60 sec; 25 cycles) using purified AcMNPV viral DNA (Summers and Smith, 1987) as template and two oligonucleotides: 5'-TTTTTTCTGCAGATGTGGGGAATCGTG-3', PstI site underlined; 5'-TTTTTTGGATCCTTGAAATTTAATGCATT-3', BamHI site

underlined. The PCR product was extracted with chloroform, precipitated in ethanol, digested with PstI and BamHI, purified from 1% agarose gel using GeneClean kit (BIOL101), and cloned into PstI/BamHI sites of pUC19 vector. The PstI/BamHI PCR insert in pUC19 and the BamHI/EcoRI fragment from the pGFP vector (ClonTech) containing the GFP gene were cloned into the PstI/EcoRI sites of pVL1392 vector (Webb and Summers, 1990). This construct was named pVL1392-24GFP (See Fig. 2, Construct #12). In this construct, the N-terminal 24 amino acid hydrophobic sequence from ODV-E25 was fused in frame with GFP. Recombinant virus expressing 24GFP is being made using the construct pvL1392-24GFP and Bsu36I digested BakPak6 viral DNA (ClonTech.). In this virus, the 24GFP fusion gene replaces the polyhedrin gene in the polyhedrin locus. Infected cells will not produce viral occlusions due to the absence of a functional polyhedrin gene.

EXAMPLE 5
STUDIES ON THE BIOCHEMISTRY AND MOLECULAR PROCESS OF TRANSPORT OF PROTEINS CONTAINING THE N-TERMINAL HYDROPHOBIC DOMAIN OF ODV-E66

Additional studies are being performed to address the molecular basis and regulation of transport of proteins containing the N-terminal amino acid hydrophobic domain of ODV-E66. These studies demonstrate the effect on both baculovirus infected insect cells and in uninfected cells including insect, vertebrate and plant cell lines. Clonal isolates of Sf9 cells that have undergone germline transformation and have a copy of the IE1-23GFP gene placed within its genome have been developed.

The results of these studies have also been observed in vertebrate and plant cell lines which have also undergone germline transformation. Additionally, wild type ODV-E66 and fusion proteins are being utilized to identify other protein factors or protein processing that may be involved in some function to assist, target, or transport proteins to membranes of the nuclear envelope.

These studies may suggest normal insect cells contain cellular cognates of several of the baculovirus ODV envelope proteins, and that these cognates may prove to be important in protein movement in uninfected cells. Sequencing of clones for two potential cellular cognates is being performed. Screening for accessory proteins that may play a role in protein transport of ODV-E66 and several other ODV envelope proteins is also being performed. Screening methods being utilized in this laboratory include: yeast two-hybrid methodology, covalent linkages of protein complexes, anti-idiotype identification of binding proteins, and identification of protein complexes using antibody isolation techniques.

EXAMPLE 6
IDENTIFICATION OF OTHER HYDROPHOBIC SEQUENCES FOUND IN BACULOVIRUS ODV ENVELOPE PROTEINS --- STRUCTURAL AND FUNCTIONAL COGNATES OF 23AA OF ODV E66

This Example is designed to identify potential cognates from other baculovirus genes with known hydrophobic sequences. The additional, known, hydrophobic sequences, found in baculovirus ODV envelope proteins, will be characterized as either structural or functional cognates of 23AA of ODV-E66. It is not known if the potential hydrophobic cognates possess the same innate ability to locate a foreign protein, or derivative in the same way, as ODV-E66 or the 23AA sequence of this invention.

Listed in Table 2 below are hydrophobic sequences identified in baculovirus ODV envelope proteins. In order to identify structural or functional cognates of the 23AA of ODV E66, constructs are being made for each of these hydrophobic sequences with protocols and goes to those disclosed above. These protocols are very similar to those designed to produce the constructs shown in Fig. 2. The analyses as shown in Example 2 are being performed to determine if a transport, retention or signal sequence, similar to the 23AA of this invention, is contained within the tested hydrophobic ODV domains.

The following abbreviations are used in Table 2 below:

ACMNPV: *Autographa californica* multinucleocapsid nuclear polyhedrosis virus

*OP*MNPV: *Orgyia pseudotasugata* multinucleocapsid nuclear polyhedrosis virus

*Bm*MNPV: *Bombyx mori* multinucleocapsid nuclear polyhedrosis virus

*Hz*SNPV: *Helicoverpa zea* singlenucleocapsid nuclear polyhedrosis virus

*Cf*MNPV: *Choristoneura fumiferana* multinucleocapsid nuclear polyhedrosis virus and

*Cp*GV: *Cydia pomonella* granulosis virus

EXAMPLE 7
APPLICATION OF NEWLY IDENTIFIED 23 AMINO ACID TARGETING SEQUENCE --- INSECTICIDES/INSECTICIDAL TOXINS

Current approaches to genetically engineer baculoviruses to serve as insecticide delivery agent(s) utilize a recombinant baculovirus for delivery of a gene encoding toxic agents to insects (Martens, 1994). For successful results, the gene of interest must be expressed, secreted from infected cells or modified by the infected cell, and become active in the insect hemolymph or cells. These approaches have resulted in the insects dying faster than when infected with wild type baculovirus. Initial indications suggest that these recombinant viruses cause sufficiently rapid cessation of feeding and will be sufficiently cost-effective to make these viral constructs commercially viable insecticidal agents in the field.

a. Insecticidal Toxins in General

Baculovirus ODV fuses with the insect gut cell to deliver their genetic components into the infected cell. The use of the 23 amino acid hydrophobic sequence of this invention, or structural or functional cognate thereof, which targets proteins to the ODV envelope, provides an effective way to directly deliver a toxin to the cellular membranes of the insect gut (See Fig. 10A for an outline). Fig. 10 is a diagram showing an overview of directed delivery to target cell and subsequent gene delivery. Fig. 10A is a diagram showing targeting and delivery to target cell via membrane fusion with the purified microvesicles or ODV envelope (I-V). If the

delivered protein has toxic activity after being incorporated into the cell membrane, then this protein may have an insecticidal application (VI). Fig. 10B is an extension of the delivery shown in Fig. 10A to include gene delivery and expression. This use is either insecticidal or therapeutic depending upon the function of the protein encoded by the recombinant gene. Thus, immediately upon infection, the direct delivery results in compromised cellular function and integrity, and cessation of eating. In addition, the direct delivery of toxin(s) to the insect gut cell membranes can be combined with the classical gene delivery approach (See Fig. 10B). The example described below is an example of one such toxin delivered as described above. Other toxins, or agents that compromise cell health, ionic pore regulators, etc. can be utilized for insecticidal purposes using the 23 AA sequence of this invention and the protein or gene delivery approach as described in this example.

b. Producing Insecticidal Toxin URF13 Protein and Direct Delivery to the Insect Midgut

Maize exhibiting the Texas male-sterile cytoplasm (cms-T) trait was widely used in commercial hybrid maize production before, in 1970, severe disease outbreaks to fungal pathogens forced the industry to curtail its use. The URF13 protein (13 kDa) encoded by the mitochondrial gene *T-urf13*, is responsible for cms-T trait and has received considerable scientific investigation because of the impact on commercial maize production (Dewey *et al.*, 1986). Unexpectedly, researchers observed that baculoviruses expressing URF13 were lethal to *Trichoplusia ni* larva (*T. ni.*; cabbage looper; Korth and Levings, 1993). Additionally, when URF13 is expressed in baculovirus and is transported to the plasma membrane of the infected insect cells, it confers T-toxin or methomyl (fungicide) sensitivity to the cell.

The ODV form of baculoviruses is the primary infectious agent in the insect. The ODV envelope fuses with the plasma membrane of the gut cells, thus immediately upon infection, viral envelope proteins are incorporated into the gut cell plasma membrane. URF13 lethality

in insects is independent of other factors and apparently only requires delivery to the plasma membrane for toxic activity.

Thus, in view of this background and discussion, the inventors propose direct delivery of URF13 to the plasma membrane of the insect gut by constructing a recombinant virus that contains URF13 in its ODV envelope (e.g., 23URF13). This direct delivery will cause damage to the insect gut cell, resulting in compromised insect health and a cessation of eating (Fig. 10A). Since URF13, when located in the plasma membrane of baculovirus infected cells, confers sensitivity to methomyl, adding trace amounts of the fungicide methomyl to the feeding formulation will increase the toxicity of the delivered protein, e.g, URF13. One advantage of this approach, is that it can be combined with the more traditional approaches to deliver gene(s) that encode additional copies of the same, or additional toxins. Thus, after the insect function is compromised, via direct delivery of a toxin to the gut, the insect will immediately be exposed to additional toxin activity, thus decreasing its ability to recover (Fig. 10B) and increasing the speed and efficacy of the insecticidal effects. To increase URF13 insecticidal activity, another mutant virus containing the 23URF13 and an additional copy of the wild type URF13 under the control of the early baculovirus promoter (IE1) may also be employed. Moreover, as will be appreciated by those skilled in this area, additional copies of either URF13 or other toxins, e.g., the scorpion toxin AaHIT, can also be used in the delivery system of this invention, although additional toxin delivery and reinforcement may be required.

The time course of delivery and concentration of any of these genes can be regulated by choice of promoter - both temporal expression, e.g., immediate early, delayed early, late or very late, and strength and activity levels of promote, as will also be understood by those skilled in this art.

EXAMPLE 8
APPLICATION OF NEWLY IDENTIFIED 23 AMINO ACID TARGETING SEQUENCE --- PROTEIN MEDIATED TARGETING AND VIRUS-LIKE PARTICLES THERAPEUTIC USE

Baculovirus infection results in a large number of small, intranuclear membrane vesicles. These have been named "microvesicles". These microvesicles serve as one source of the ODV envelope and it has been shown that the 23 amino acid sequence locates proteins to these structures at a high efficiency (Hong *et al.*, 1994, Braunagel *et al.*, 1996a, Braunagel *et al.*, 1996b; Hong *et al.*, 1996). Techniques are currently being tested to purify these microvesicles. The inventors have identified at least one protein that results in a greatly enhanced production of intranuclear microvesicles, if expressed at high levels in an infected cell (Braunagel and Summers, unpublished observations). Thus, purification of large quantities of microvesicles from infected cells *in vitro*, appears possible. For the purposes presented here, purified microvesicles and "virus-like" particles are used interchangeably. Thus, by inserting proteins(s) that are of medical value into these vesicles, virus-like particles useful themselves as a therapeutic are created.

a. Virus-Like Particle Delivery of Protein to Gut Tissue

Baculovirus ODV fuses with insect gut cells (Kawanishi *et al.*, 1972). There is no evidence that ODV randomly fuses with other membrane systems. Of interest, ODV is virtually non-infectious when delivered into the bloodstream or is used as an infectious agent *in vitro* (Volkman and Summers, 1977). The inventors have evidence that the baculovirus envelope contains at least one protein homolog in its viral envelope that resembles a protein of human rotavirus, a virus which infects humans through the gut (Braunagel *et al*, 1992; and unpublished observations). It was therefore predicted that ODV would fuse with mammalian gut membranes. The development of recombinant virus carrying defined fusion proteins, e.g., 23GFP, in the ODV envelope now provides the tools to confirm this prediction *in vitro*.

Specific therapeutic or regulatory proteins, and derivatives thereof, may now be incorporated directly into the microvesicles and directly delivered to the microvillar membranes of the gut tissue upon ingestion. Thus, generating 23AA-fusion products of integral membrane proteins selectivity incorporates the fusion proteins into the microvesicles. Upon ingestion, these proteins become transiently incorporated in gut microvillar membranes. Proteins and/or receptors that enhance or replace essential components for nutrient and/or drug absorption, are examples of this type of delivery.

Thus, it now appears feasible that suitable foodstuff or drug formulations can include microvesicles or artificially prepared structural or functional cognates containing the relevant accessory protein for proper absorption and/or delivery. This provides an effective direct delivery system to the intestinal microvillar membranes in a mammalian system.

b. Virus-Like Particle Delivery Into the Bloodstreams

A number of approaches have been designed to expose certain antigens, and/or receptors or anti-receptors to infectious agents in the bloodstream. One well characterized example is delivery of CD4. CD4 is a receptor that resides on a subset of T cells and is a target for the anti-receptor gp120 of Human Immunodeficiency Virus (HIV). The recognition of CD4, by gp120, allows viral entry and subsequent viral infection (Maddon *et al.*, 1986). If CD4 is presented to the HIV virus on another surface than T cells, it acts as a "negative sink" or scavenger of the virus, thus decreasing the amount of virus that successfully infects the target cells. This strategy has been studied and shows potential as an AIDS therapeutic (Nicolau *et al.*, 1990; Arvinte *et al.*, 1989 and Webb *et al.*, 1989).

While the concept of this type of delivery system is straightforward, the methods of incorporating integral membrane proteins, *i.e.*, receptors, with a defined secondary structure and orientation into artificial membrane systems (*ie.* liposomes) or resealed ghosts can be very difficult. Usually only a small

proportion of the total incorporated protein is exposed and conformationally correct. Thus, only a small percent of protein is functional. The advantage of using baculovirus derived microvesicles is that the orientation of the fusion protein can be designed and tested to determine functional authenticity. Once the construct is tested and confirmed to be biologically active, all copies of the protein should be inserted into the microvesicles in the same conformation. Additionally, multiple proteins or protein complexes can be inserted into the microvesicles. Use of different promoters of various strengths and temporal activities, allows for the regulation of the amount and timing of gene expression and protein production (Belyaev and Roy, 1993). Thus, this approach provides significant control and flexibility not only of conformation of protein insertion, but also with a way to engineer active protein complexes in membrane vesicles or virus-like particles for therapeutic use. This approach is feasible in both animal and human hosts.

Many different proteins can be genetically engineered to be inserted into intranuclear microvesicles. Since ODV is not the infectious form of baculovirus in cell culture and is not required for passage *in vitro*, then, presumably, any number of proteins could be incorporated into the ODV envelope for this purpose. Since the production of infectious ODV is not required, some non-essential ODV proteins could be deleted from the viral genome to generate additional space in the ODV envelope for recombinant, fusion proteins. For example, it is already known that ODV-E66 and ODV-E56 are non-essential for viral propagation *in vitro* (Hong, 1995; Braunagel *et al.*, 1996a).

EXAMPLE 9
APPLICATION OF NEWLY IDENTIFIED 23 AMINO ACID TARGETING SEQUENCE --- PROTEIN MEDIATED TARGETING AND GENE OR DRUG DELIVERY

a. General Approach

Using the observation that any protein could be targeted to the ODV envelope, and that baculovirus can be used to reconstruct membrane protein complexes, including specific fusion proteins, it becomes possible to insert a receptor, etc. into the ODV envelope that is specific for cell type recognition and fuses only with the target cell. A virus that contains such engineered protein(s) on its viral envelope could be used in several ways: (1) Direct delivery to the target cells via specific recognition and fusion, thus direct delivery of the protein of interest to the target cell membrane; and (2) Use of specific protein complexes to direct and fuse ODV envelope to the target cell with subsequent gene delivery using any promoter recognized by the host cell. In this manner, the baculovirus becomes a specific vector to target protein (ie. receptor and/or drug) or gene delivery in both animal and human hosts. (See Fig. 10 for overview).

b. Delivery of Protein(s) to Cellular Plasma Membranes Using ODV as Agent

This example (*i.e.*, using modified ODV to deliver proteins to the membranes of the target cell) is similar to the example using purified virus-like particles to deliver proteins, but has distinct advantages. In an occlusion positive baculovirus, mature ODV is incorporated into viral occlusions. Once ODV is embedded within the viral occlusion, it is very stable. This form of the virus remains viable for an extended period of time, several years under outdoor environmental conditions, and considerably longer if stored protected from UV light. Viral occlusions are easily purified from infected cell lysates. The occlusion can be dissolved using an alkaline treatment to release ODV.

Since refrigeration and drug storage is a major problem in many areas of the world, this invention can be used to transport therapeutic agent(s) in the stable form of viral occlusions. At the site of delivery, a premeasured vial of occlusions is mixed with an aliquoted alkaline solution, allowed to incubate at room temperature for a specific period of time, thereby releasing the ODV, then neutralized with the appropriate solvent system and administered to the patient.

c. Gene and/or Drug Delivery to Target Cell Using ODV as Agent

This example utilizes the ability to direct ODV to the target cell by incorporating receptors, fusion proteins etc. into the ODV envelope. Upon fusion with the host cell membrane the viral nucleocapsid is released into the host cell. When genes encoding therapeutic agents are genetically engineered into the viral genome under the control of promoters that are recognized by the host cell (ie. baculovirus IE1 gene promoter), then this system can be utilized for gene delivery purposes. The obvious example uses gp120 and the AIDS virus. Engineering ODV to have a 23-CD4 receptor located on the viral envelope, enables the targeting of that virus directly to HIV infected cells. After membrane fusion with the HIV infected cell, a gene that is under the control of an appropriate promoter encoding an anti-HIV toxic protein could be delivered and expressed. The resultant protein production would mediate the killing of the HIV infected cell.

Baculovirus infection and DNA replication have been analyzed in a number of species. These included: intraperitoneal injection of virus into white mice (Ignoffo, 1971); feeding virus to rats, white mice and guinea pigs (Ignoffo *et al.*, 1972; Ignoffo and Heimpel, 1965) and inoculating mammalian cell lines with virus (Ignoffo and Rafajko, 1972; Roder and Punter, 1977). Over the past 25 years, a large body of literature has been collected that confirms the safety of wild type baculoviruses. The overwhelming conclusion from this literature with inoculation of a large number of hosts with

baculoviruses, is that exposure of non-host cells to baculoviruses does not result in cytopathic, or detrimental effects (Groner, 1986).

If baculoviruses are utilized as delivery agents to mammalian hosts, safety issues would need to be carefully addressed. Again, baculovirus offers unique advantages. If baculovirus is incorporated into some non-host cells, limited viral gene expression occurs (IE1 promoter) but the entry of the virus does not result in late gene expression or productive infection. The genome of *Autographa californica* and *Bombyx mori* nuclear polyhedrosis virus (AcMNPV; BmMNPV) has been sequenced (Ayres *et al.*, 1994; and GenBank #L33180) and the genes essential for viral DNA replication have been identified (Kool *et al.*, 1994). If viral DNA replication occurring in the non-host, target cell is identified as a safety issue, it should be possible to place one or more of the genes required for DNA replication under the control of an inducible promoter (temperature sensitive, chemical induction etc.). In this manner, the virus can be replicated in the laboratory using inducing conditions, and will not be capable of replication in the target cell.

EXAMPLE 10
APPLICATION OF 23 AMINO ACID TARGETING SEQUENCE - - GENETIC GERM LINE TRANSFORMATION OR DELIVERY OF PROTEIN TO VERTEBRATE AND/OR INVERTEBRATE CELLS

This example takes into account several potential properties of this novel 23AA targeting sequence.

Another use that combines many of the features already described and extends their application is to selectively deliver a gene that has been engineered to allow germline transformation of the target cell genome. This approach would include: Proteins within the ODV envelope that serve to target the recombinant virus to the specific cell; The gene of interest is modified within the baculovirus genome to contain flanking transposable elements, retroviral transposition elements, pantropic vectors, etc. such that after delivery, the engineered gene becomes incorporated into the cellular genome (Jarvis *et al.*, 1996; Matsubara *et al.*, 1996).

Another use for such targeting and gene delivery system might include precise delivery of a gene to a cell type lacking a normal function. By placing the gene of interest in the baculovirus genome using transposable elements according to this invention, the gene, upon transposition, becomes stably integrated into the genome of the target cell.

Signal targeting to the nuclear membrane into a vertebrate is also envisioned. This application takes into account several potential properties of the cell by any one of a number of viral vectors (adenovirus, herpesvirus, retrovirus adeno-associate virus, etc.) or cell transformation systems to transform the cell for the novel targeting sequence, including constitutive expression and insertion of 23AA - X protein for whatever function desired.

If the nuclear membrane insertion signal functions as the data shows, then the targeting sequence could be inserted into the nuclear envelope of any cell (vertebrate, invertebrate, or lower eukaryotes) by a variety of transformation or vector systems. An example might include a sindbis virus transformation vector carrying an insecticidal or lethal 23 - X protein into mosquito cells.

EXAMPLE 11
APPLICATION OF THE 23 AMINO ACID TARGETING SEQUENCE -- PROTEIN TARGETING

Data show that proteins containing the 23 amino acid sequence locate to the nuclear envelope, both the outer and inner nuclear membrane, during defined times of the cell cycle. This location has been shown in both insect (Sf9) cell lines and mammalian (COS) cell lines during mitosis (as indicated by bi-nucleated cells). Thus, in actively dividing cells, the fusion protein is located in the nuclear membrane; while in non-dividing cells, the fusion protein is incorporated within a general, but undefined set of cytoplasmic membranes. By using targeted ODV gene delivery, it becomes possible to specifically deliver a gene using the modified ODV envelope containing targeting proteins, express a gene encoding a drug or

toxin and deliver that drug or toxin to the nuclear membrane of actively dividing cells. This approach provides another level of molecular approaches to potentially control or modify the organization and function of the nuclear envelope.

a. Control of Cell Cycle

Using either gene delivery or germline transformation approaches, it may be possible to target cells which are undergoing mitosis. An example might include selective targeting of a cancerous tissue growth. All of the cells of that tissue type contain the same defining surface receptor, so normal and non-cancerous cell would be targets for the modified ODV. All cells would receive the gene encoding the therapeutic or toxic agent, however the therapeutic effects would only be active in dividing cells, when the protein located to the nuclear membrane. In this manner, cancerous cells, which are dividing at an accelerated rate, would be primary recipients of the therapeutic agent or toxic agent.

Examples of places in the cell mitotic event that may lend itself to interruption include: not allowing nuclear membrane dissolution (ie. through interrupting the lamin B phosphorylation pathway) or adding a copy of cyclin B that has been genetically modified so that it cannot be ubiquinated and thus freezing the cell in metaphase (Hunter, 1993). These are two such examples, however there are many processes and temporal activities within the cell cycle that potentially could be altered or modified by foreign protein localization to the nuclear envelope and specifically the inner nuclear membrane.

b. Delivery of Proteins to the Nuclear Membrane For Therapeutic Use

By using protein targeting, germline transformation techniques and/or gene delivery approaches, proteins could be delivered to the inner nuclear membrane that may function to interfere with or modify the effects of or functions of, known oncogenes. Both approaches, in above Examples 11(a) and 11(b), are potential uses of this technology

to deliver proteins to the nuclear membrane of cancerous cells for cancer therapy. However, this approach should not be limited to cancerous conditions. Any malfunction in a cell that could be corrected or treated with gene/protein delivery to the nuclear membrane would be a candidate for use of this technology.

EXAMPLE 12
APPLICATION OF 23 AMINO ACID TARGETING SEQUENCE - - DIAGNOSTICS

The instant invention can be used to produce a variety of different proteins that locate to the ODV envelope. Using the 23 amino acid sequence of this invention would be especially beneficial for producing diagnostics based on receptors or integral membrane protein or protein complexes. Briefly, the advantages of this system are: (a) proteins can be placed in the ODV envelope in a known orientation; (b) ODV can be generated in insect larva rather than the more expensive *in vitro* systems; and (c) the ability to incorporate ODV into viral occlusions at will allows ease of purification of viral occlusions and ODV, and ability to store the preparation for extended periods of time. Viral occlusions are easy to purify and allow for long-term storage in that form.

The ability to produce certain diagnostic reagents in insect larva allows the protein reagent(s) to be produced more efficiently, inexpensively and with less effort compared with producing proteins from tissue culture systems. Fig. 11 is a diagram comparing the levels of protein expression *in vitro* (tissue culture cells) with the levels in larva for several proteins (applicable to diagnostic applications). Protein can be produced *in vitro* and in insect larva utilizing recombinant baculovirus at high levels.

As can be seen for certain proteins, the expression level can be much higher in larva than in tissue culture cells. For example, one insect larvae can provide enough material for approximately 30,000 ELISA diagnostic tests. Another advantage of isolating viral occlusions from larva (and not from tissue culture cells) is that the

virus and incorporated proteins are extremely stable in the occlusion form. Once viral occlusions have been purified, they can be stored for years before final purification of ODV and sample use.

* * *

The chart shown on Fig. 13 outlines many of the potential applications for the N-terminal 23 amino acid (23AA) sequence as described in the preceding Examples.

* * *

Other embodiments are within the following claims.

* * *

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The following references may facilitate understanding or practice of certain aspects of the present invention. Inclusion of reference in this list is not intended to and does not constitute an admission that the reference represents prior art with respect to the present invention.

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What is claimed is:

1. A substantially pure DNA encoding a transport polypeptide, the DNA comprising a strand which hybridizes at high stringency to a probe comprising a sequence of at least 15 consecutive nucleotides as shown in Fig. 1B, the polypeptide sharing at least 80% sequence identity with a series of corresponding amino acids listed in Fig. 1B.

2. The DNA of claim 1, wherein the transport polypeptide is a polypeptide, 23AA, which directs localization of a desired protein to occlusion derived virus envelopes.

3. A vector comprising the DNA of claim 2.

4. The DNA of claim 2, wherein the DNA is operably linked to regulatory sequences for expression of the protein, the regulatory sequences comprising a suitable promoter.

5. A cell comprising the DNA of claim 4.

6. An essentially homogeneous population of cells, each of which comprises the DNA of claim 4.

7. A substantially pure transport polypeptide, wherein the polypeptide:

(a) contains a hydrophobic domain; and

(b) localizes to occlusion derived virus envelopes thereby directing the localization of fused or hybrid proteins to occlusion derived virus envelopes.

8. The polypeptide of claim 7, the polypeptide sharing at least 80% sequence identity with the amino acids listed in Fig. 1B.

9. An antibody which specifically binds to an epitope of the polypeptide of claim 7, or an antigen-binding fragment of the antibody.

10. The antibody or antibody fragment of claim 9, wherein the antibody or antibody fragment is linked to a detectable label.

11. An antigenic fragment of a polypeptide having the amino acids identified in Fig. 1B.

12. A substantially pure DNA comprising a sequence of at least 15 consecutive nucleotides of the region from nucleotides 122 to 190, inclusive, of Fig. 1A.

13. The substantially pure DNA of claim 12 comprising a sequence of at least 15 consecutive nucleotides of the region encoding the transport polypeptide of Fig. 1A.

14. A method of directing a desired protein to occlusion derived virus envelopes comprising the steps of:

(a) constructing a vector comprising a marker protein and the DNA of claim 2;

(b) fusing a nucleotide sequence encoding a desired protein to the DNA of Step (a) to form a hybrid vector;

(c) incubating the hybrid vector of Step (b) with a biological sample; and

(d) determining the localization of the marker protein within the biological sample, wherein localization indicates the presence of occlusion derived virus envelopes and also delivery of the hybrid vector to the envelopes.

15. A method of detecting 23AA transport polypeptide in a biological sample comprising contacting the biological sample with the antibody or antibody fragment of claim 9 and determining whether the antibody or antibody fragment binds to a component of the sample, the binding being an indication that the sample contains 23AA transport polypeptide having a hydrophobic domain.

16. Isolated DNA having a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of Fig. 1B; and

(b) nucleotide sequences which, through the degeneracy of the genetic code, encode the same peptide gene product as that encoded by the nucleotide sequence of Fig. 1B.

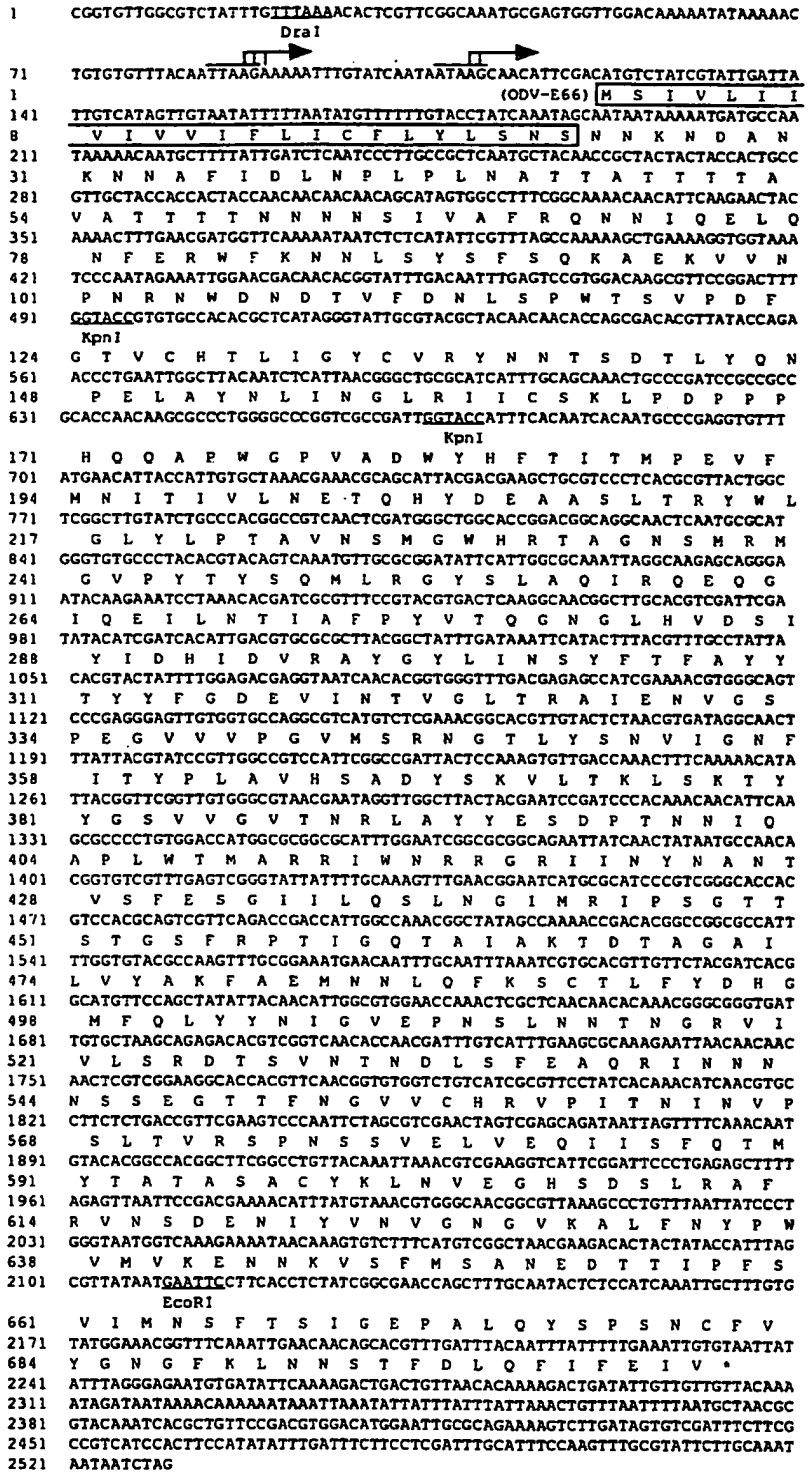


FIG 1a

Fig. 1B

NUCLEOTIDE AND AMINO ACID SEQUENCE FOR 23AA:

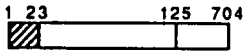
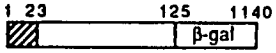
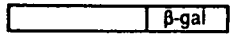
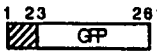
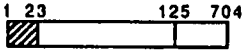
```
A T G  T C T  A T C  G T A  T T G  A T T  A T T  G T C
M      S      I      V      L      I      I      V

A T A  G T T  G T A  A T A  T T T  T T A  A T A  T G T
I      V      V      I      F      L      I      C

T T T  T T G  T A C  C T A  T C A  A A T  A G C
F      L      Y      L      S      N      S
```

AMINO ACID SEQUENCE FOR 23AA:

M S I V L I I V I V V I F L I C F L Y L S N S

	Name	Structure	Promoter	Gene Locus	Production of Viral Occlusion	Protein Location
1.	ODV-E66		ODV-E66	ODV-E66	Yes	ODV-Env, M, NE
2.	125β-gal		ODV-E66	ODV-E66	Yes	ODV-Env, M, NE
3.	Δ2-23β-gal		ODV-E66	ODV-E66	Yes	–
4.	23β-gal		ODV-E66	ODV-E66	Yes	ODV-Env, M, NE
5.	pAcUW21-23GFP		p10	polyhedrin	Yes	ODV-Env, M, NE, CM
6.	pVL1393-23GFP		polyhedrin	polyhedrin	No	ODV-Env, M, NE, CM
7.	pVL1393-E66F		polyhedrin	polyhedrin	No	ODV-Env, M, NE, CM
8.	pAcUW21-23URF13		p10	polyhedrin	Yes	ODV-Env, M, NE, CM
9.	pIE1HR4-23GFP (uninfected Sf9 cells)		IE1	N/A	N/A	Cell cycle regulation to NE
10.	pCMV-23GFP (uninfected COS-1 cells)		CMV-IE	N/A	N/A	Cell cycle regulation to NE
11.	pRTL2-23GFP (Plant cells)		35S	N/A	N/A	?
12.	pVL1392-24GFP (ODV-E25)		polyhedrin	polyhedrin	?	?

N-terminal 23 amino acid hydrophobic sequence derived from ODV-E66

N-terminal 24 amino acid hydrophobic sequence derived from ODV-E25

ODV-Env - ODV envelope
M - Intranuclear microvesicles and membrane structures
NE - Nuclear envelope
CM - Cytoplasmic membranes
CMV-IE - Major immediate early promoter of cytomegalovirus
N/A - Not applicable

FIG 3

FIG 3a

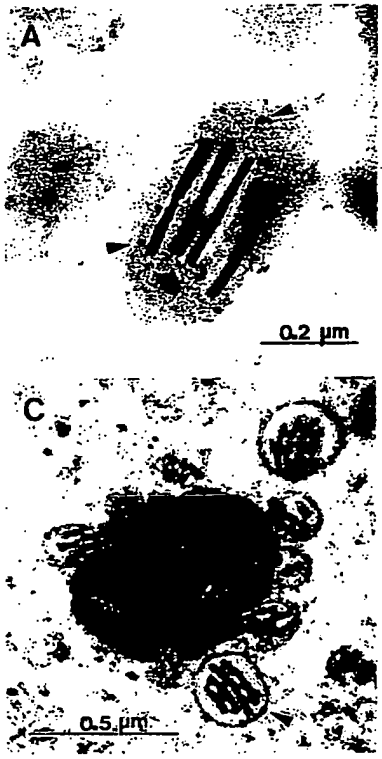


FIG 3c

FIG 3b

5/14 FIG 4

FIG 4a

FIG 4b

FIG 4c

FIG 5

FIG 5a

FIG 5b

7/14
FIG 6 a-i

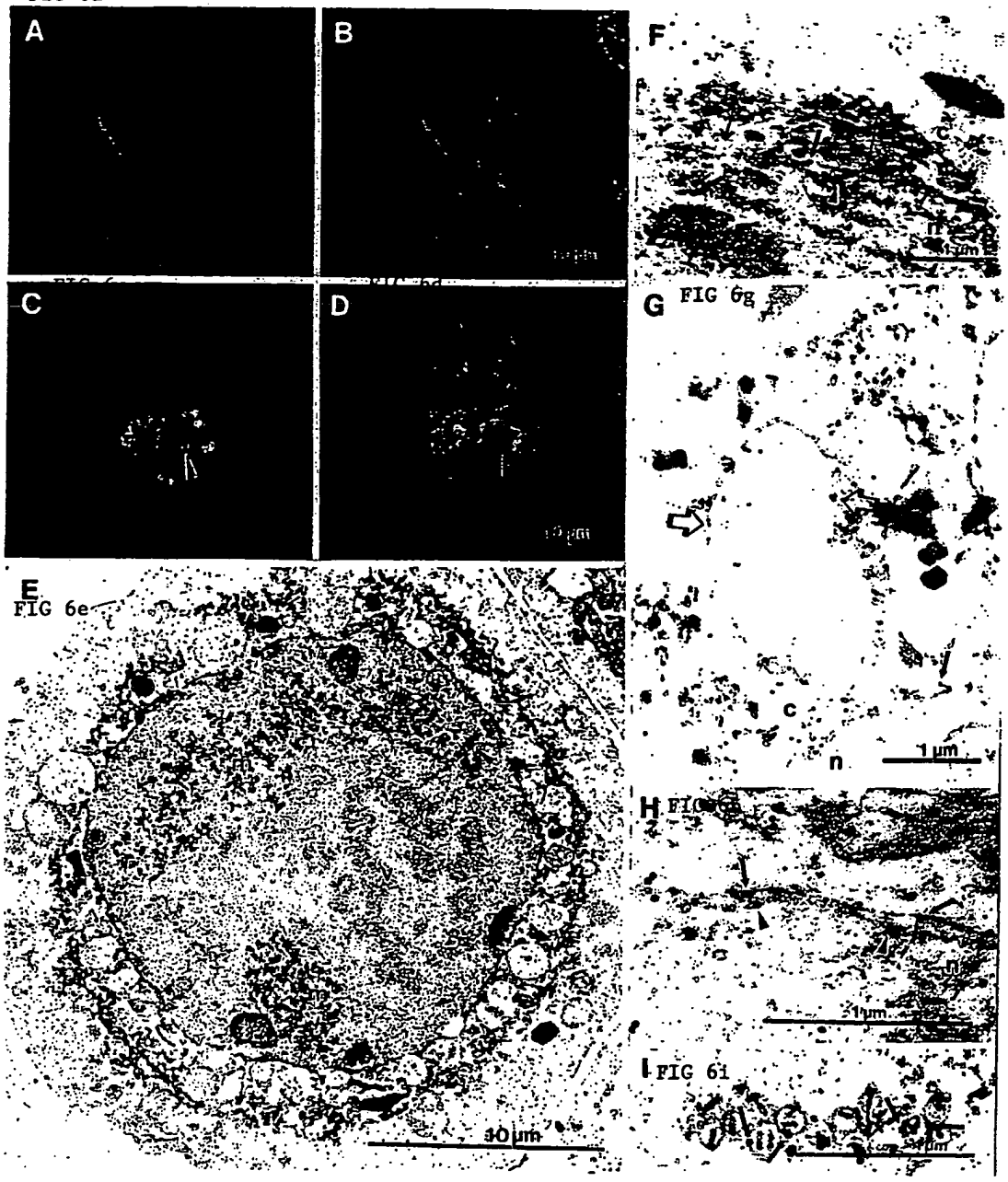


FIG 6a
FIG 6b
FIG 6f
A
B
C
D
E
FIG 6e
30 µm
F
G
FIG 6g
c
n
1 µm
H
FIG 6h
n
1µm
I
FIG 6i

FIG 7a

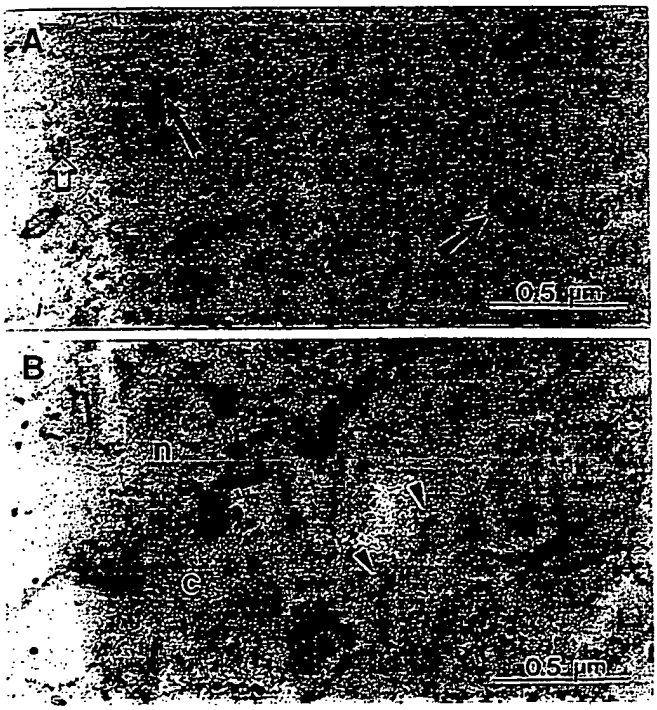


FIG 7b

FIG 8a-h

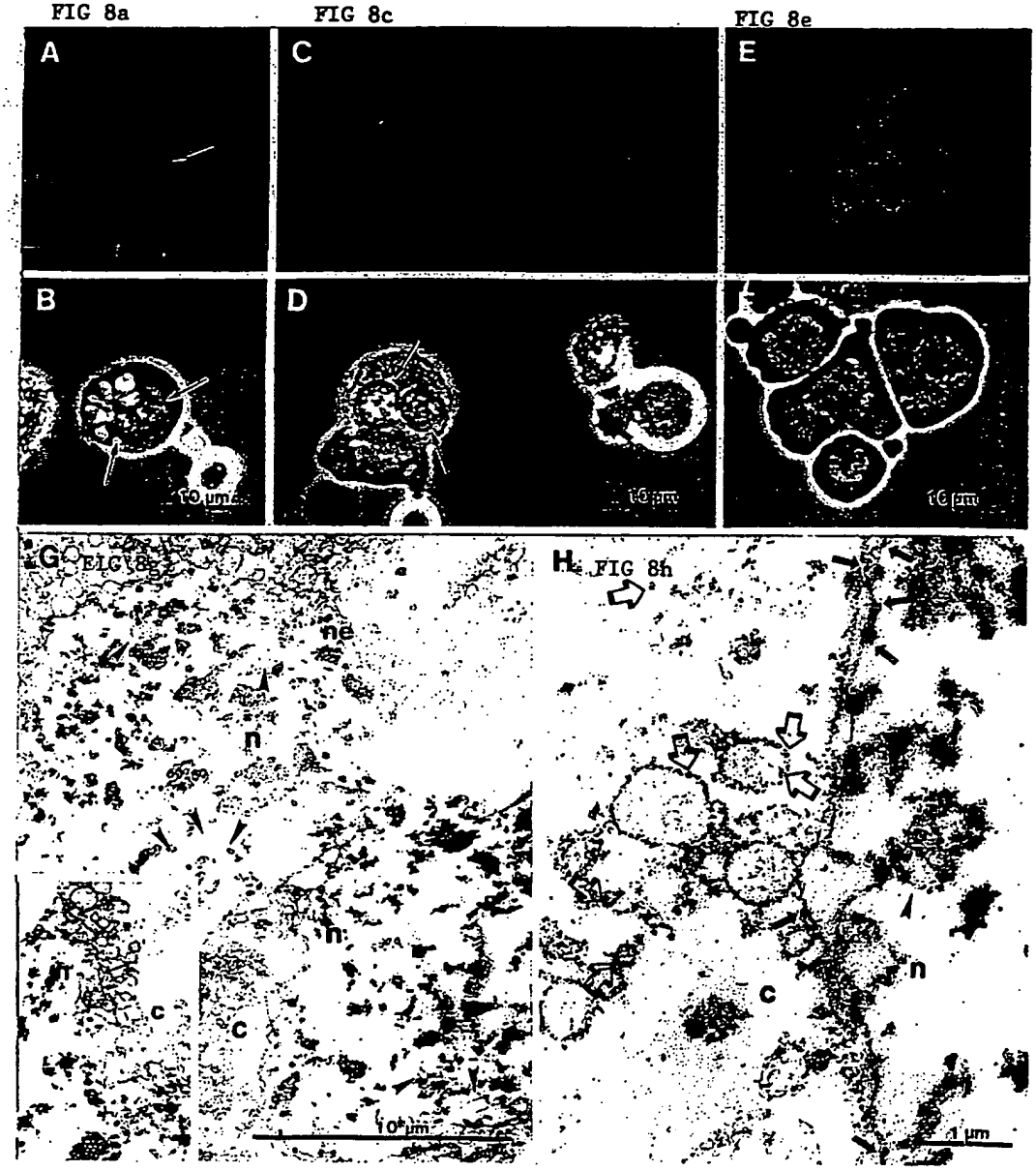
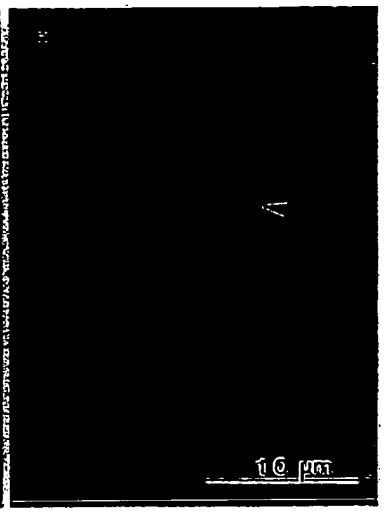


FIG 8a
FIG 8c
FIG 8e
A
C
E
B
D
F
10 µm
10 µm
10 µm
G FIG 8g
H FIG 8h
ne
n
n
n
c
c
c
10 µm
1 µm

FIG 9

FIG 9a

FIG 9b



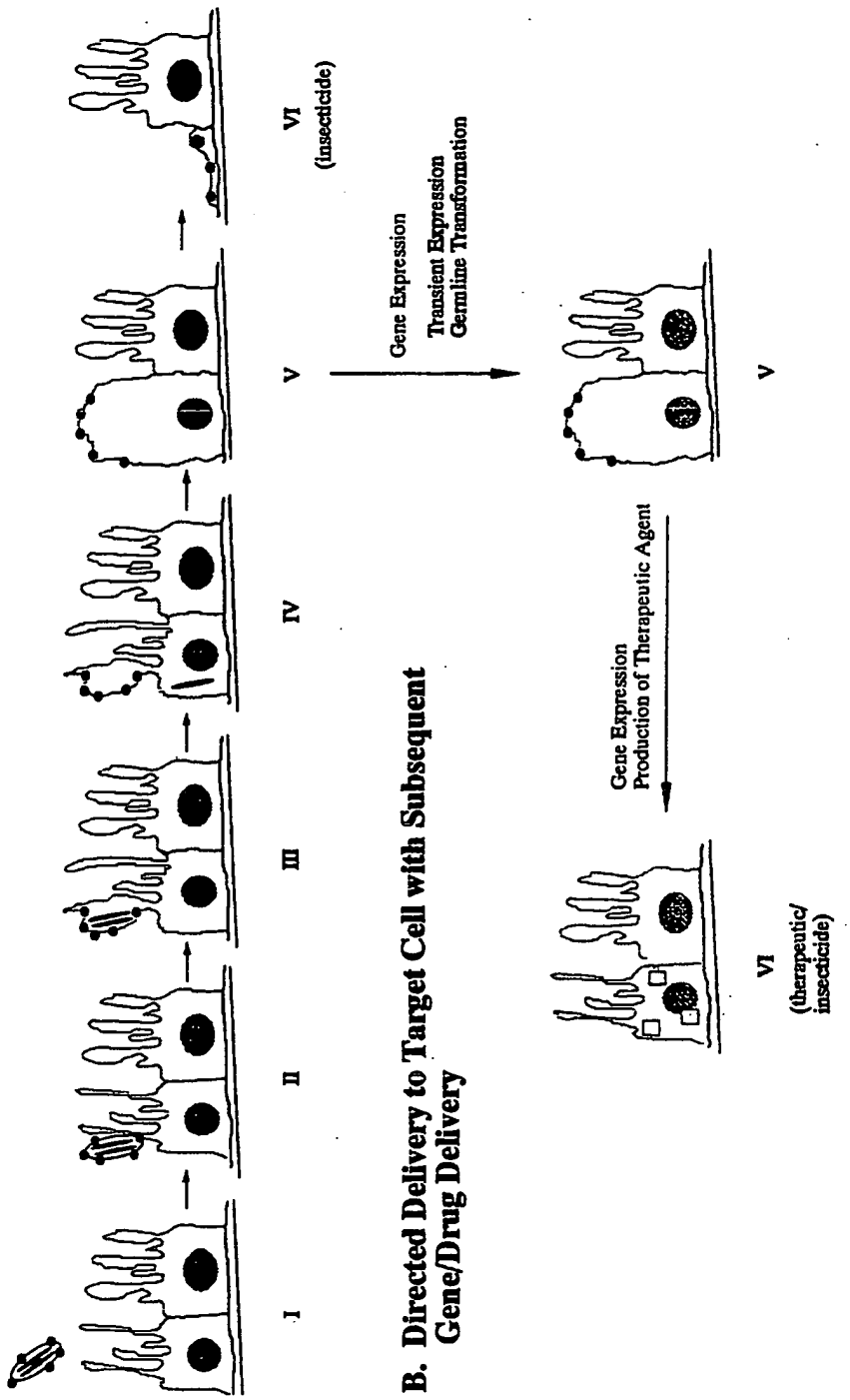
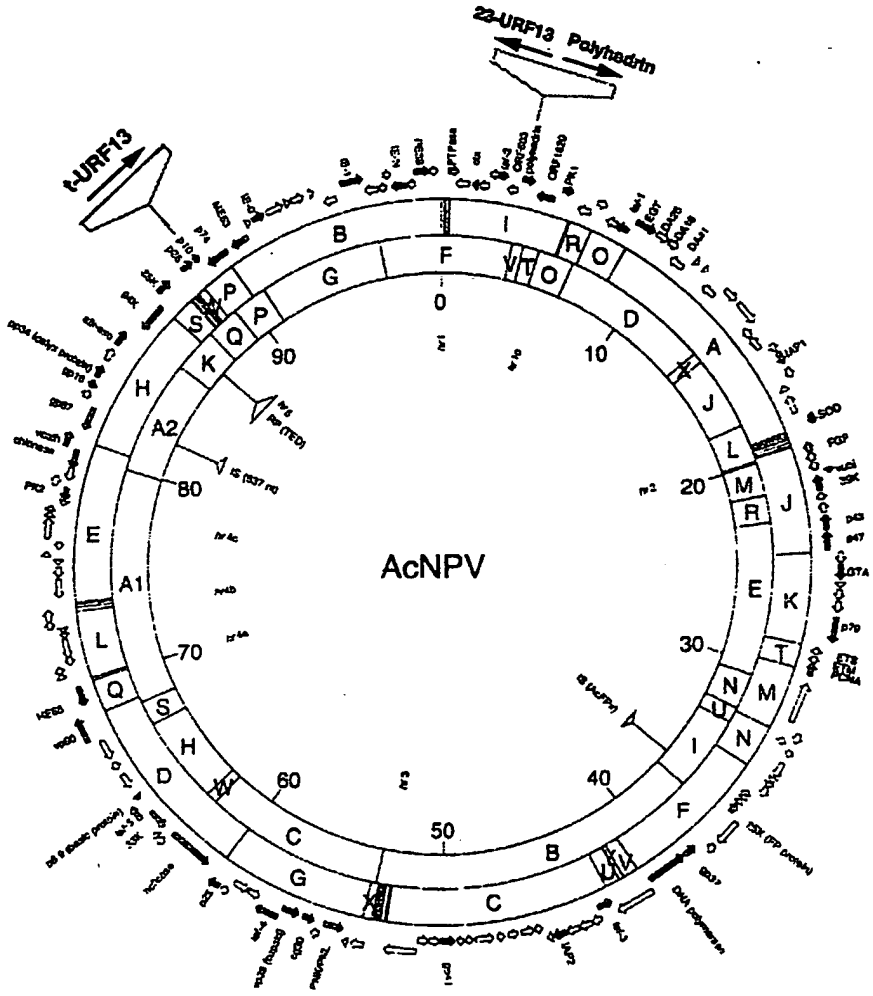

A. Directed Delivery to Target Cell
I
II
III
IV
V
VI
(insecticide)
Gene Expression
Transient Expression
Germline Transformation
B. Directed Delivery to Target Cell with Subsequent Gene/Drug Delivery
Gene Expression
Production of Therapeutic Agent
V
VI
(therapeutic/
insecticide)

FIG 10

FIG 11

LARVAL-TISSUE CULTURE EXPRESSION LEVELS

	Tissue Culture	Larval	References
Firefly luciferase	*S. littoralis* >25% total protein	*T. ni* 15% total protein	Jha *et al.*, 1990
IgG2A	*T. ni* 6.4 µg/ml supernatant	*T. ni* 800 µg/larva/7 dpi	Reis *et al.*, 1992
Murine retinoic acid receptor	Sf9 cells 300 pmol/mg protein	*Manduca sexta* 100 pmol/mg protein	Ross *et al.*, 1992
VSV-N protein	Sf9 cells 41-60% total protein	*Spodoptera exigua* 68% total protein larva = 30,000 ELISAS	Ahmad *et al.*, 1993
Rinderpest virus N protein		*Spodoptera frugiperda* larva = 15,000 sera tests	Ismail *et al.*, 1994
Insulin-like growth factor II		silkworm larva 3.6 mg/larva	Marumoto *et al.*, 1987
Human α interferon		silkworm larva 50 µg/hemolymph/larva	Maeda *et al.*, 1985
Bacterial luciferase		*E. acrea* 195 µg/larva	Richardson *et al.*, 1992



Genomic map of *Autographa californica* NPV. Multiple genes can be inserted at both the polyhedrin and p10 locus. In this manner, recombinant virus can be constructed that are occlusion +, and contain both 23-URF13 and WT t-URF13. (Ayres *et al.*, 1994)

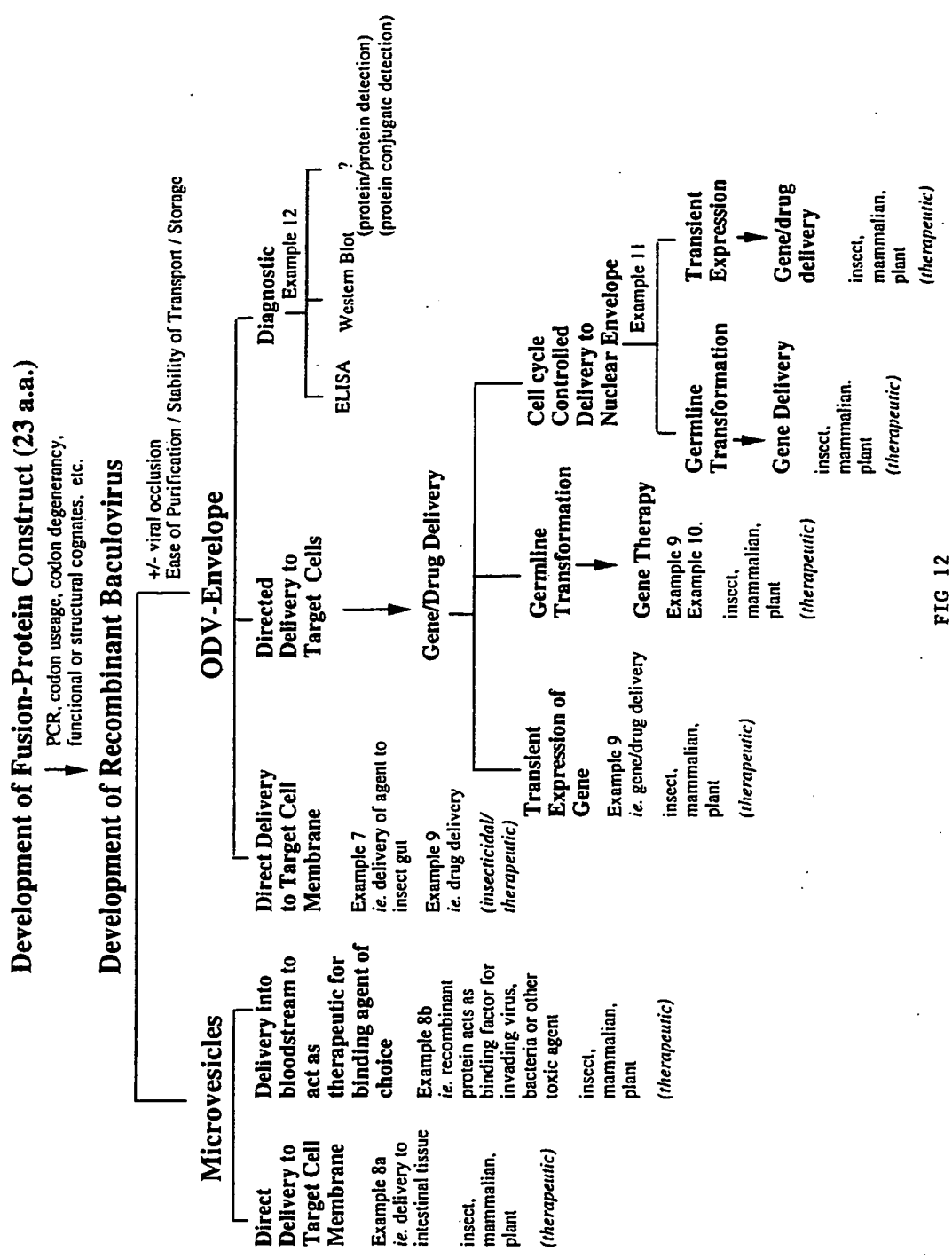

Development of Fusion-Protein Construct (23 a.a.)
PCR, codon useage, codon degeneracy, functional or structural cognates, etc.
Development of Recombinant Baculovirus
+/- viral occlusion
Ease of Purification / Stability of Transport / Storage
Microvesicles
ODV-Envelope
Direct Delivery to Target Cell Membrane
Example 8a ie. delivery to intestinal tissue
insect, mammalian, plant
(therapeutic)
Delivery into bloodstream to act as therapeutic for binding agent of choice
Example 8b ie. recombinant protein acts as binding factor for invading virus, bacteria or other toxic agent
insect, mammalian, plant
(therapeutic)
Direct Delivery to Target Cell Membrane
Example 7 ie. delivery of agent to insect gut
Example 9 ie. drug delivery
(insecticidal/ therapeutic)
Directed Delivery to Target Cells
Gene/Drug Delivery
Transient Expression of Gene
Example 9 ie. gene/drug delivery
insect, mammalian, plant
(therapeutic)
Germline Transformation
Gene Therapy
Example 9
Example 10.
insect, mammalian, plant
(therapeutic)
Cell cycle Controlled Delivery to Nuclear Envelope
Example 11
Germline Transformation
Gene Delivery
insect, mammalian. plant
(therapeutic)
Transient Expression
Gene/drug delivery
insect, mammalian, plant
(therapeutic)
Diagnostic
Example 12
ELISA
Western Blot
?
(protein/protein detection)
(protein conjugate detection)

FIG 12